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Patent
Attorney's Docket No. 016914-039

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	**MAIL STOP MISSING PARTS**
Thorsteinn LOFTSSON et al.	Group Art Unit: 1623
Application No.: 10/750,940	Examiner:
Filed: January 5, 2004	Confirmation No.: 2037
For: HIGH-ENERGY CYCLODEXTRIN COMPLEXES	

LETTER ACCOMPANYING FILING OF DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

The subject application is a continuation of Appln. No. 09/250,185, filed February 16, 1999, now U.S. Patent No. 6,699,849 B1. This continuation was filed without a copy of the inventors' declaration/power of attorney, provoking issuance of a Notice to File Missing Parts. The Missing Parts (copy of the inventors' declaration/power of attorney) and requisite surcharge are being filed concurrently herewith. Also filed herewith are copies of four declarations under 37 C.F.R. § 1.132 which were filed in the parent and which applicants request be considered by the Examiner herein. In addition, applicants submit herewith a new declaration under 37 C.F.R. § 1.132 executed on January 26, 2004 by Mar Masson, Ph.D., one of the present inventors. The experiments described in this declaration provide NMR proof of the ring-opening mechanism reflected in applicants' claims. The Examiner of the parent application, Examiner L. E. Crane, did not find the earlier-submitted declarations (of which copies are enclosed) sufficiently convincing proof of the ring-opening mechanism to permit the ring-opening mechanism to be reflected in the claims therein, although applicants thought the earlier declarations more than adequate. However, Examiner Crane

specifically requested NMR proof be submitted before he would allow the claim language to reflect the ring-opening. Now that the NMR data is submitted herein in the January 26, 2004 declaration of Dr. Masson, it is believed that there are no remaining obstacles to allowance of the language of the claims herein.

It is realized that the present claims overlap with those granted in the parent. In the event that the Examiner is otherwise willing to allow the claims in this application, it is requested that he/she phone the undersigned so that an appropriate terminal disclaimer can be promptly filed.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

Date: May 21, 2004

By: Mary Katherine Baumeister
Mary Katherine Baumeister
Registration No. 26,254

P.O. Box 1404
Alexandria, Virginia 22313-1404
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Patent Application
Attorney's Docket No. 016914-039

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	
	)	
Thorsteinn LOFTSSON et al.	)	Group Art Unit:
	)	
Application No.:	)	Examiner:
	)	
Filed: January 5, 2004	)	Continuation of U.S. Patent Application
	)	No. 09/250,185, filed February 16,
For: HIGH-ENERGY CYCLODEXTRIN COMPLEXES	)	1999, now allowed
	)	

DECLARATION OF MAR MASSON
PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, MAR MASSON, declare as follows:

1. That I am a citizen of Iceland residing at Fjolnisvegur 1, 101 Reykjavik, Iceland.

2. That I graduated from the University of Iceland in 1987 with a B.S. in chemistry, from the Copenhagen University in 1990 with a Cand. Scient. Chemistry, from Japanese studies at the Tokyo Institute of Technology, Foreign Student Training Center in 1991, and from the Tokyo Institute of Technology in 1995 with a Ph.D. in Biotechnology.

3. That in 1995, I was employed by the University of Iceland, Faculty of Medicine, Department of Biochemistry, as a researcher; from 1995 to 1997, I was employed by the University of Iceland, Faculty of Medicine, Department of Pharmacy, in research for Professor Thorsteinn Loftsson, Ph.D.; and in 1997, I was employed by the

University of Iceland, Faculty of Medicine, in a post-doctoral position supervised by Professor Thorsteinn Loftsson, Ph.D. and Professor Einar Stefansson, M.D., Ph.D.

4. That from 1998 to the present, I have been employed by the University of Iceland, Faculty of Pharmacy, as Associate Professor in Medicinal Chemistry.

5. That I am one of the inventors of the above-referenced patent application.

6. That I am familiar with the prosecution history of U.S. Application No. 09/250,185, filed February 15, 1999, of which the present application is a continuation.

7. That I am making this declaration to address the Examiner's position in the parent that further proof by means of NMR data was needed in order for the Examiner to allow claims reflecting the ring-opening mechanism.

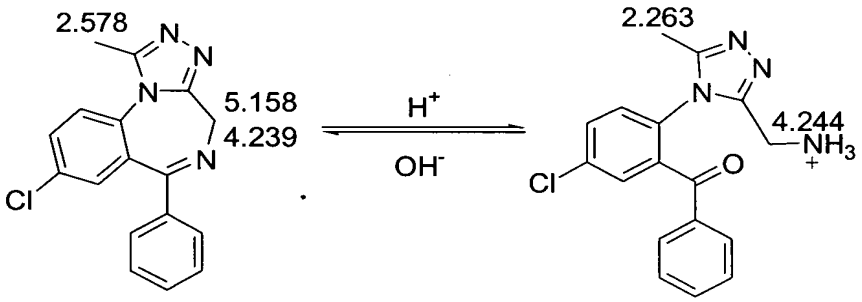
8. That I designed and oversaw the following NMR study to provide the proof of the ring-opening mechanism requested by the Examiner (although I already considered sufficient proof of the ring-opening mechanism to have been submitted in declarations filed in the parent application).

Experimental

^{1}H and ^{13}C NMR spectra were taken at 25 ^{o}C on a Varian INOVA 400 MHz and a Varian INOVA 500 MHz NMR spectrometer respectively, using a 5 mm sample tube. D_2O was used as a solvent; the water signal was used as an internal reference for ^{1}H NMR. Chemical shifts were expressed in parts per million (ppm) relative to those of the HOD signal (4.700 ppm). No internal reference was used in order to avoid interference with the binding to cyclodextrins.

Several series of alprazolam and midazolam samples were prepared with either 10% (w/v) hydroxypropyl-β-cyclodextrin (HPβCD) or 10% (w/v) β-cyclodextrin sulfobutyl ether (SBECD) at different pH. The samples were sonicated at elevated temperature, and shaken overnight at room temperature to reach equilibrium. The ^{1}H NMR spectra were recorded and the ratio of opened form vs closed form was determined by integrating the representative peaks from both opened and closed forms in each sample. The pH value of each sample was measured right after the NMR experiment. All data entries were summarized in the graph presented later in this report.

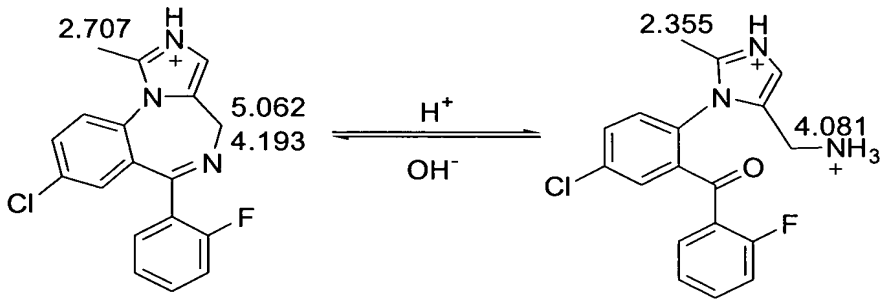
The ^{1}H NMR spectra of alprazolam were recorded in acidic D_2O to establish the accurate assignment of the chemical shifts of protons in closed form (**1A**) and opened form (**1B**).



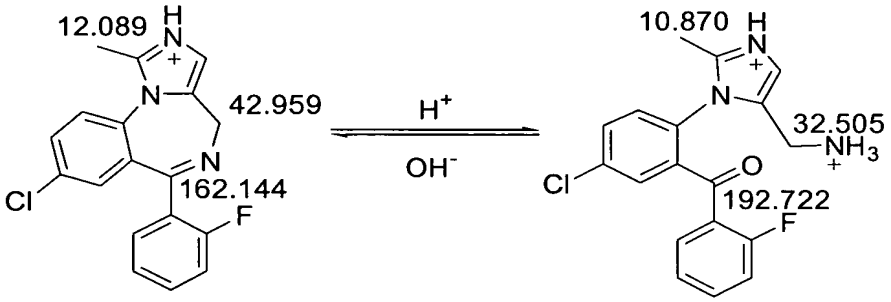
1A **1B**

In the ^{1}H NMR spectrum (Figure 1), the nonequivalent protons of **1A** appeared at 5.158 and 4.239 ppm. These doublets merged into a quartet at 4.244 ppm of **1B** when DCl was added to **1A** and the resulting sample was shaken overnight at room temperature. In addition, the singlet at 2.578 ppm, which corresponds to the methyl group on the triazole moiety, underwent an upfield shift to 2.263 ppm in **1B**.

Since the solubility of midazolam in aqueous solution is greater than alprazolam, not only the ^{1}H NMR but also ^{13}C NMR spectra of midazolam were recorded in acidic D_2O to establish the accurate assignment of the chemical shifts of protons and carbons in closed form (**2A**) and opened form (**2B**).



2A **2B**



2A **2B**

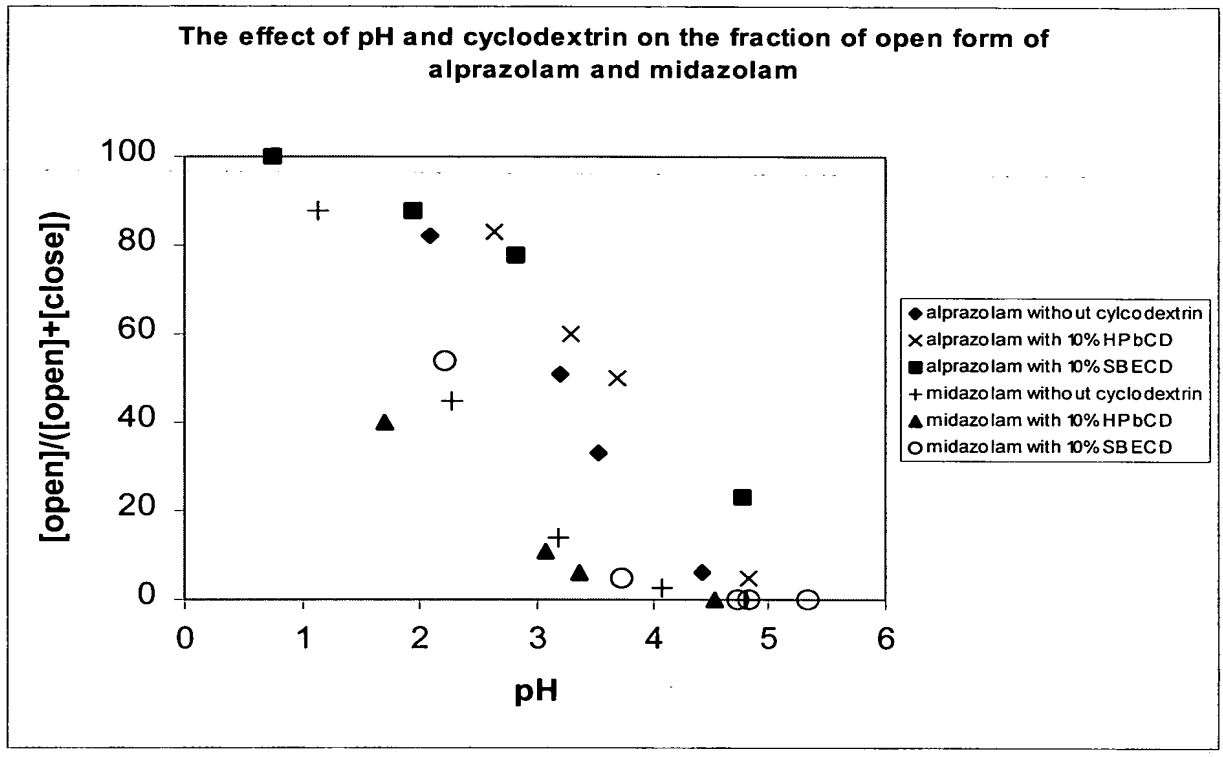
In the ^{1}H NMR spectrum (Figure 2), the nonequivalent protons of **2A** appeared at 5.062 and 4.193 ppm. When DCl was added to **2A** and the sample was shaken overnight at room temperature, these doublets merged into a singlet at 4.081 ppm of **2B** whose imidazole ring is relatively basic (pK_a 6.9) compared to the triazole ring ($pK_a \leq 1.5$) in alprazolam. In addition, the singlet at 2.707 ppm, which corresponds to the methyl group on the imidazole moiety, underwent an upfield shift to 2.355 ppm in **2B**. Changes in the ^{13}C NMR spectrum of midazolam (Figure 3) that occurred upon addition of DCl provided

more direct evidence of the reaction shown above. A downfield shift took place for the imine carbon, from 162.144 to 192.722 ppm, supporting the presence of a benzophenone carbonyl carbon. The upfield shift of a methylene carbon from 42.959 to 32.505 ppm was also in accordance with the ring-opening reaction. As expected, the signal corresponding to the methyl group on the imidazole moiety changed very little with an upfield shift from 12.089 to 10.870 ppm.

When both **1B** and **2B** were treated with sodium deuteroxide, the ring closed again to afford **1A** and **2A**.

The ^{1}H NMR expansion of aromatic region of mixture of **2A** and **2B** was examined carefully and led to the accurate assignment of doublets at 7.93 and 7.91 ppm to **2B**, 7.79 and 7.77 ppm to **2A** respectively (Figure 4). This spectrum was compared with spectra of mixture of **2A** and **2B** complexing with SBECD and HPβCD. The double doublet at 7.9 ppm of **2B** experienced a downfield shift to 8.1 ppm to give a multiplet, while the double doublet at 7.8 ppm of **2A** also went downfield to 7.95 ppm to yield a multiplet in the sample of mixture of **2A** and **2B** plus SBECD, indicating the complex formation of SBECD with **2A** and **2B**. The double doublet at 7.9 ppm of **2B** experienced a downfield shift to 8.0 ppm to give a multiplet, while the double doublet at 7.8 ppm of **2A** also went downfield to 7.88 ppm to yield a doublet in the sample of mixture of **2A** and **2B** plus HPβCD, indicating the complex formation of HPβCD with **2A** and **2B**. The major difference is that the complexed proton in closed form in HPβCD is doublet while the same complexed proton in closed form in SBECD is multiplet, indicating the structural difference in geometry of the two complexes.

Graph:



9. That the NMR data conclusively show:

(a) the ring-opened form of the benzodiazepines is formed in the cyclodextrin complexation medium;

(b) the ring-opened form will complex at a pH below about 5; and

(c) the fraction of ring-opened form increases when the pH is lowered (cf. the graph set forth above).

10. That the NMR results detailed herein verify and correlate well with the data shown in Table 3 of the specification (also Table 3 of the parent application) showing the general effect of pH and complexation on the presence of the ring-opened form of two representative benzodiazepines, midazolam and alprazolam, complexed with two

representative cyclodextrins, hydroxypropyl-β-cyclodextrin and β-cyclodextrin sulfobutyl ether.

11. That the NMR data presented here are consistent with the HPLC data presented in the parent, particularly that discussed in the second 37 C.F.R. § 1.132 declaration of Thorsteinn Loftsson and the second 37 C.F.R. § 1.132 declaration of Mar Masson submitted during prosecution of parent Application No. 09/250,185.

12. That this declaration together with the four declarations submitted during the prosecution of parent Appln. No. 09/250,185 therefore justify the allowance of claims which reflect the ring-opening mechanism, such as Claims 1-65 as filed in this application.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 26.01.2004

MAR MASSON

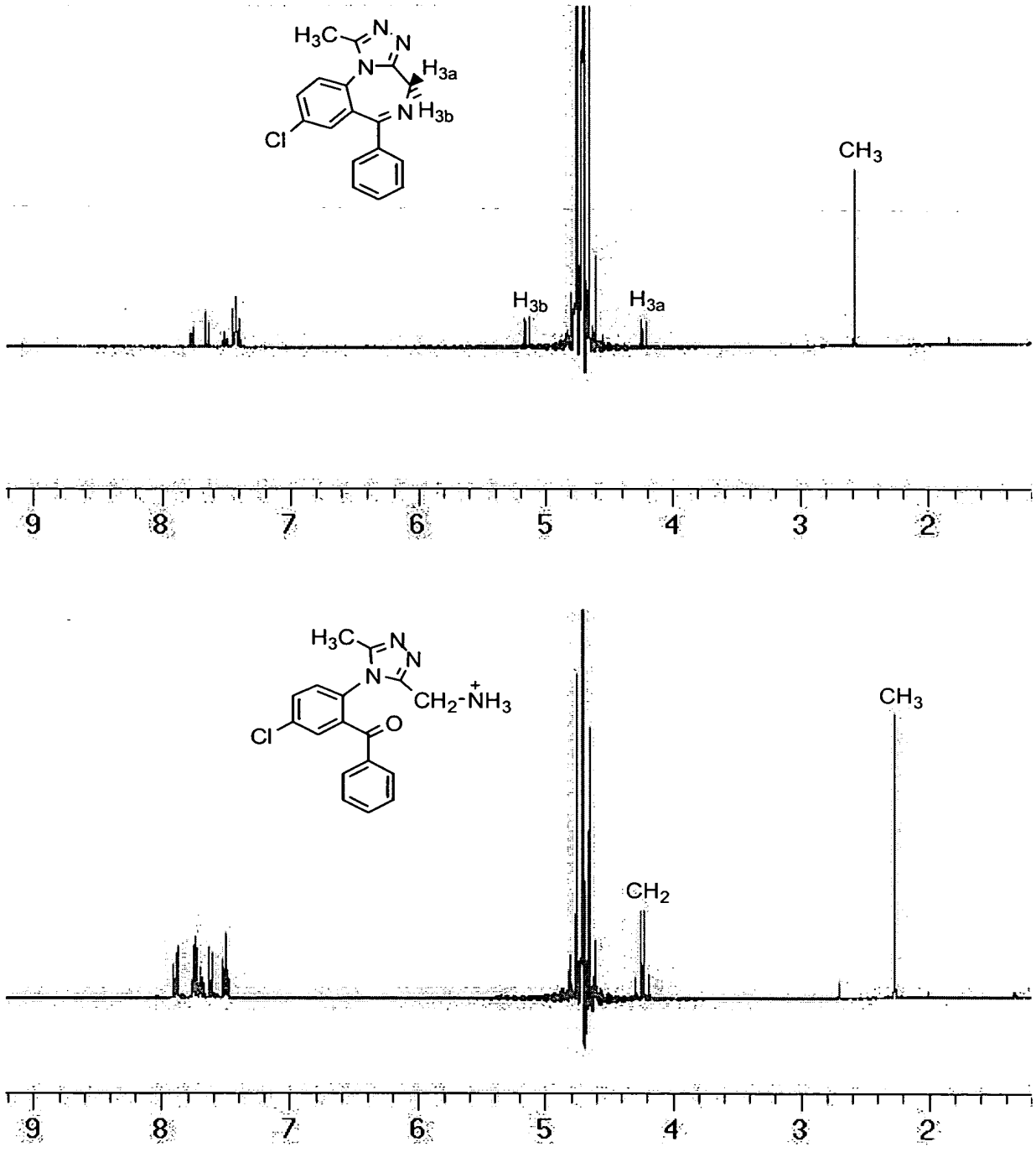


Figure 1. ^{1}H NMR spectra of **1A** (top) and **1B** (bottom).

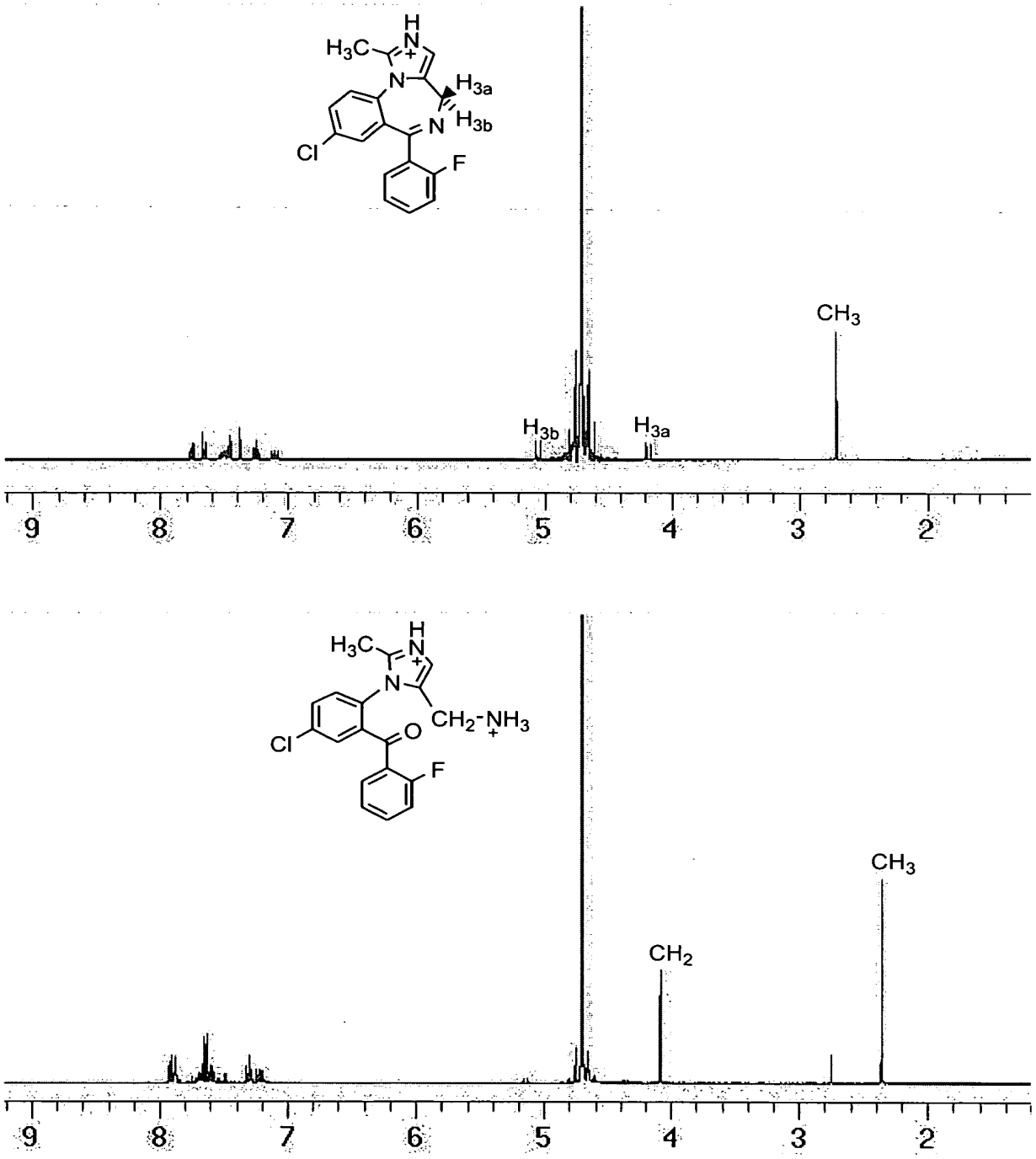


Figure 2. ^{1}H NMR spectra of **2A** (top) and **2B** in major (bottom).

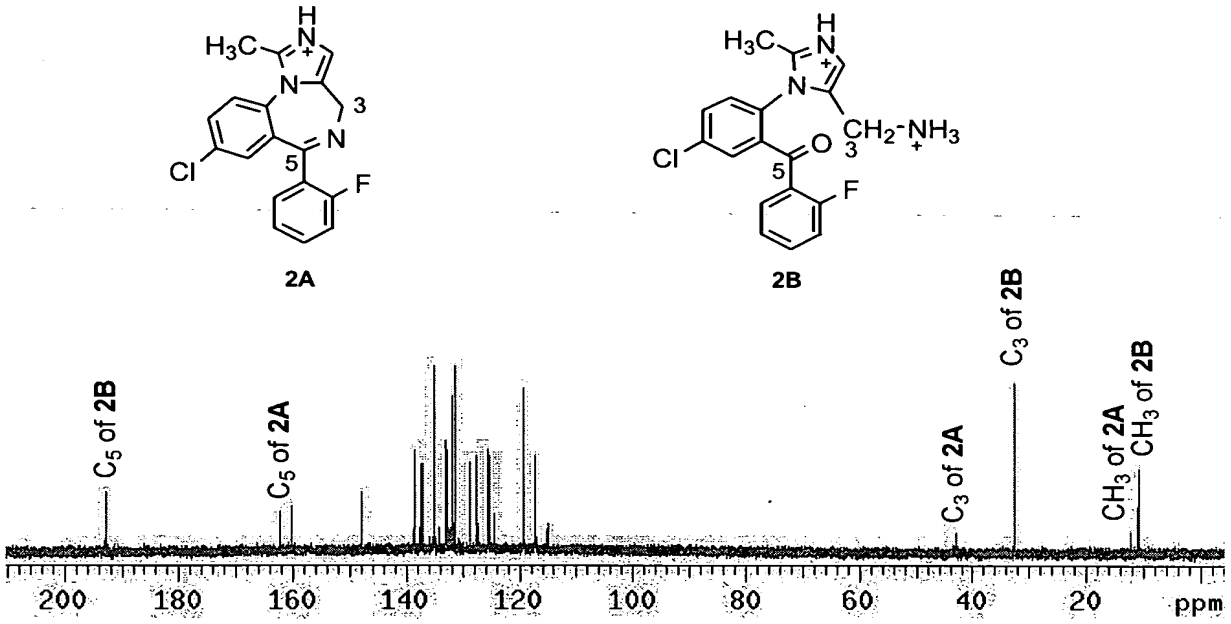


Figure 3. ^{13}C NMR spectrum of **2A** (minor) and **2B** (major).

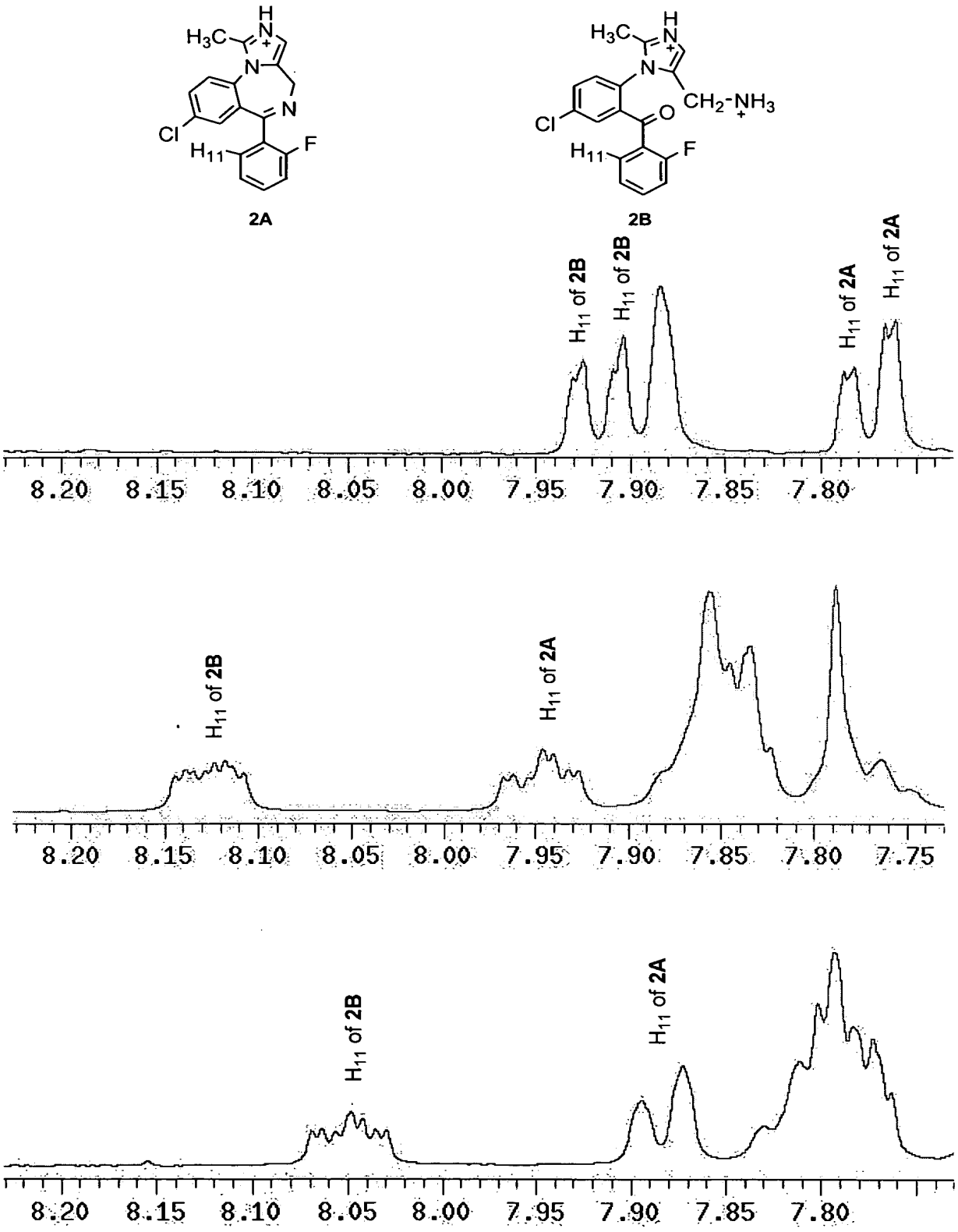


Figure 4. ^{1}H NMR spectra of the expansion of aromatic region of **2A** + **2B** (top), **2A** + **2B** + 10% SBECD (middle), and **2A** + **2B** + 10% HPβCD (bottom).

COPY PROVIDED FOR APPLN. NO. 10/750,940
(016914-039)

Patent Application
Attorney's Docket No. 016914-007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Thorsteinn LOFTSSON et al	Group Art Unit: 1623
Application No.: 09/250,185	Examiner: Lawrence E. Crane
Filed: February 16, 1999	Confirmation No.: 1768
For: HIGH-ENERGY CYCLODEXTRIN COMPLEXES	

SECOND DECLARATION OF MAR MASSON PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, MAR MASSON, declare as follows:

1. That I am a citizen of Iceland residing at Fjolnisvegur 1, 101 Reykjavik, Iceland.

2. That I graduated from the University of Iceland in 1987 with a B.S. in chemistry, from the Copenhagen University in 1990 with a Cand. Scient. Chemistry, from Japanese studies at the Tokyo Institute of Technology, Foreign Student Training Center in 1991, and from the Tokyo Institute of Technology in 1995 with a Ph.D. in Biotechnology.

3. That in 1995, I was employed by the University of Iceland, Faculty of Medicine, Department of Biochemistry, as a researcher; from 1995 to 1997, I was employed by the University of Iceland, Faculty of Medicine, Department of Pharmacy, in research for Professor Thorsteinn Loftsson, Ph.D.; and in 1997, I was employed by the

University of Iceland, Faculty of Medicine, in a post-doctoral position supervised by Professor Thorsteinn Loftsson, Ph.D. and Professor Einar Stefansson, M.D., Ph.D.

4. That from 1998 to the present, I have been employed by the University of Iceland, Faculty of Pharmacy, as Associate Professor in Medicinal Chemistry.

5. That I am one of the inventors of the above-referenced patent application.

6. That I am familiar with the prosecution history of this application, including the prior art relied upon by the Examiner.

7. That I am familiar with the contents of the accompanying SECOND DECLARATION OF THORSTEINN LOFTSSON PURSUANT TO 37 C.F.R. §1.132.

8. That I am making this declaration to address the Examiner's uncertainty as to the existence of the ring-opening mechanism operative here and his request for spectroscopic evidence, as well as to address the Examiner's belief that the specification should describe the precise means used by applicants to detect the ring-opened form (apparently based on the Examiner's belief that one of ordinary skill would not know how to detect the ring-opened form based on the information in the application itself).

9. That with respect to the ring-opening mechanism, the accompanying SECOND DECLARATION OF THORSTEINN LOFTSSON PURSUANT TO 37 C.F.R. §1.132 shows that two forms of benzodiazepines investigated can be present in acidic cyclodextrin solutions saturated with the drug. Analysis of these solutions by reverse phase HPLC chromatography shows that one relatively lipophilic form will have a retention time identical to the retention time of the benzodiazepine dissolved in an organic solvent. Thus any person having ordinary skill in the art will conclude that this peak can be assigned to

the original "ring-closed" form of the drug. The second peak is a more hydrophilic degradation product with a relatively short retention time. When the benzodiazepine solution is diluted into mobile phase containing organic solvent, then the intensity of the peak for the hydrophilic degradation product will decrease and the intensity of the peak for the ring-closed form will increase in a relatively slow process, which can take a couple of hours (also shown in Tables 5, 6 and 7 in the original application), until all the drug is in the ring-closed form. Thus, it is shown that in acidic aqueous solutions there is a "slow" equilibrium between the "ring-closed" form and a relatively hydrophilic product of the benzodiazepine that is produced in aqueous acidic solutions. We have maintained that the hydrophilic product is the ring-opened form of the benzodiazepines. It has been reported that benzodiazepines can degrade through ring opening and that this reaction can be reversible; see M.R. Dobrinska, Diazepam, in K.A Connors, G. L. Amidon, and V. J. Stella, *Chemical Stability of Pharmaceuticals, a Handbook for Pharmacists*, John Wiley & Sons, New York 1986; copy appended. Any person with ordinary skill in the art will understand that two components of a chemical equilibrium can only be separated by HPLC if the chemical equilibrium is much slower than the intra-phase equilibrium between the mobile phase and the stationary phase in the HPLC column. It is also clear, to anyone with ordinary skill in the art, that this is not the case for ionization or the formation of transient forms such as hydrated imines. The hydrophilic product can therefore only be the "ring-opened" form of the drug as no alternative and plausible explanation can be offered to explain the presence of this peak.

10. That with respect to the Examiner's mention of the lack of spectroscopic evidence, I fail to see how spectroscopic data would better support our conclusion than the data from the chromatography. Interpretation of spectroscopic data is, like interpretation of chromatographic data, based on the current knowledge of chemical theory. First we must propose some structures that are consistent with current chemical theory and can be detected with the analytical method. Then we can see that only some (or one) structure(s) can explain the experimental data. The HPLC data can only be explained by proposing the presence of a "ring-opened" form of the benzodizepines. No other explanation, that is consistent with current chemical theory, can be given to explain the HPLC data.

11. That with respect to the Examiner's complaint that the present application does not describe the precise means used by applicants to detect the ring-opened form, a step which is now reflected by some of the claims, implying that one of ordinary skill would not know how to detect the ring-opened form, I state the following:

The application clearly teaches that the ring-opened form is produced and is detected, as set forth in Table 3 of the application as originally filed. This is enough information alone to teach one of ordinary skill how to detect the ring-opened form. In other words, the ordinary skilled worker would immediately know how to detect the ring-opened form once he is taught that it exists. Thus, aware that the ring-opened form exists from the teachings of the application, the person of ordinary skill knows, merely from the structures of the ring-opened and ring-closed forms, that they can be separated; from Example 3 and Table 3 of the application, the skilled worker knows what amounts of specific ring-opened forms to expect for specific benzodiazepine/cyclodextrin

complexations at specific pHs. Once the skilled worker knows to look for it, and knows from the examples in the application, including Example 3, to allow sufficient time for equilibration before looking for it, and in light of his knowledge of what the properties of a ring-opened form are from its very structure (i.e. considerably more hydrophilic than the ring-closed form), he can readily select a method for separating the two forms or, in other words, detecting the presence of the ring-opened form. There is nothing exotic or out of the ordinary, for example, to the selection of a reverse phase HPLC system as described in the SECOND DECLARATION OF THORSTEINN LOFTSSON PURSUANT TO 37 C.F.R. §1.132. Other methods of detection are also within the skill in the art. What was not within the skill in the art was the realization that complexation of benzodiazepines with cyclodextrins can be enhanced by means of the ring-opened form. The very existence of the ring-opened form in benzodiazepine/cyclodextrin complexation was not even known prior to applicants' invention.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 23/4/2003

MAR MASSON

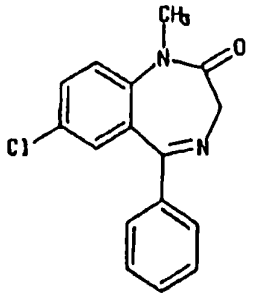
Diazepam

GENERAL

Names

Diazepam; 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one.

Structure



$C_{16}H_{13}ClN_2O$

mol. wt. 284.74

Forms Available

Diazepam.

Physical Properties

Off-white to yellow, practically odorless, crystalline powder; melting point 131 to 135°C. Solubility: 0.05 mg/mL in water, 17 mg/mL in propylene glycol, 41 mg/mL in 95% alcohol, 220 mg/mL in benzene (1). pK_a = 3.3 at 20°C (2).

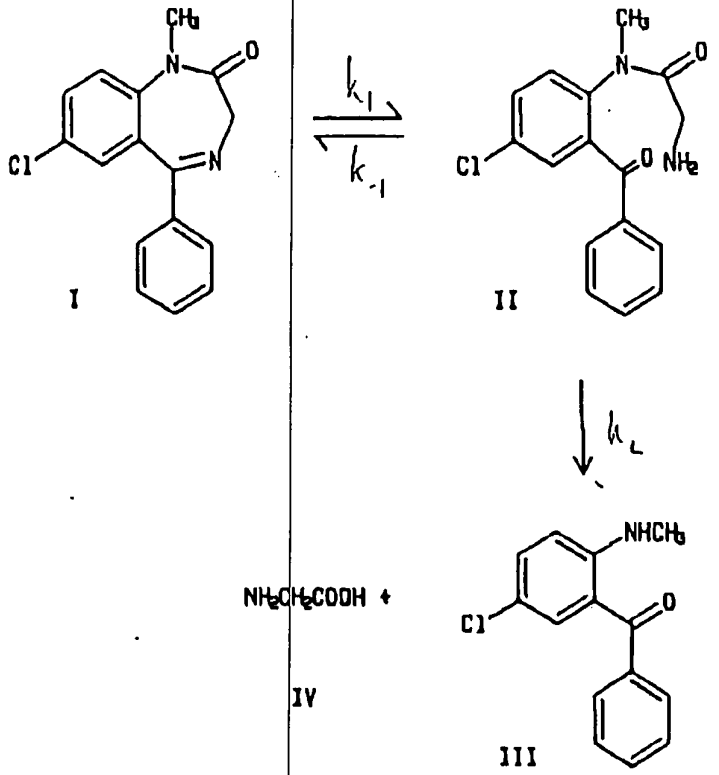
Stability Summary

Diazepam is one of the more stable substituted 1,4-benzodiazepines. It undergoes hydrolysis in aqueous solution, with the benzophenone as the major decomposition product. Maximum stability toward hydrolysis occurs around pH 5. The solubility and stability of diazepam are enhanced in mixed aqueous solvent systems consisting of propylene glycol, or polyethylene glycol, and ethanol, benzyl alcohol, and water. Low-moisture-content diazepam tablets and suppositories, stored for extended time periods, show minimal decomposition.

DRUG KINETICS

Reactions and Rate Equations

In aqueous solution, diazepam (I) undergoes hydrolysis via an open-ring intermediate leading to the formation of 2-methylamino-5-chlorobenzophenone (III) and glycine (IV) (3-6):



The structure of the intermediate as proposed by Han et al. (3), resulting from cleavage of the 4,5-azomethine bond of diazepam, has been supported by Nakano et al. (5) using an authentic sample of II [2-glycyl(methyl)amino-5-chlorobenzophenone]. This reaction is reversible and pH dependent. At pH values less than the pK_a the degradation kinetics of diazepam are biphasic; at higher pH values recyclization of II is facile and the degradation kinetics are monophasic in character (3,5).

At pH values below the pK_a of diazepam, the first reaction step can be described (3) by

$$k = k_{H_2O} f_{HS} \qquad (2)$$

or its kinetic equivalent:

$$k = k_H[H^+]f_s \qquad (3)$$

The second reaction step, as well as the single reaction at pH values above the pK_a, is attributed (3) to hydrolysis of the intermediate (II) according to

$$k = k_H[H^+]f_{HS} + k_{H'}[H^+]f_s + k_{OH}[OH^-]f_s \qquad (4)$$

where the observed rate constant k is defined in terms of the bimolecular rate constants (k_H, $k_{H'}$, k_{H_2O}, k_{OH}), the catalyzing species (H^+, OH^-), and the fractions of protonated or deprotonated diazepam or intermediate II (f_{HS}, f_s). The second term in Eq. (4), specific acid catalysis of the deprotonated intermediate, is kinetically equivalent to uncatalyzed hydrolysis of the protonated intermediate.

pH-Rate Profile

The pH-rate profile for the hydrolysis of diazepam at 80°C is given in Figure 1; both reaction steps are shown (3). For the first reaction step (labeled k_1), the discontinuity observed at pH values above the pK_a is attributed to facile recyclization of II, it being in its free-base form (3,5). The pH-rate data were fit with Eq. (2) with $k_{H_2O} = 1.5 \times 10^{-3}$ s^{-1}. The pH-rate data for the second step (labeled k_2), and for the single reaction step at higher pH values, were fit with Eq. (4) with values of $k_H = 7.5 \times 10^{-5}$ M^{-1} s^{-1}, $k_{H'} = 1.8 \times 10^{-3}$ M^{-1} s^{-1}, and $k_{OH} = 1.3 \times 10^{-3}$ M^{-1} s^{-1}. The observed first-order rate constant reported for diazepam hydrolysis at 80°C was $<1.7 \times 10^{-7}$ s^{-1} over a pH range of 4.0 to 7.9 (3) corresponding to a half-life of over 47 days. Mayer et al. (4) reported maximum stability of diazepam in aqueous solution at approximately pH 5.

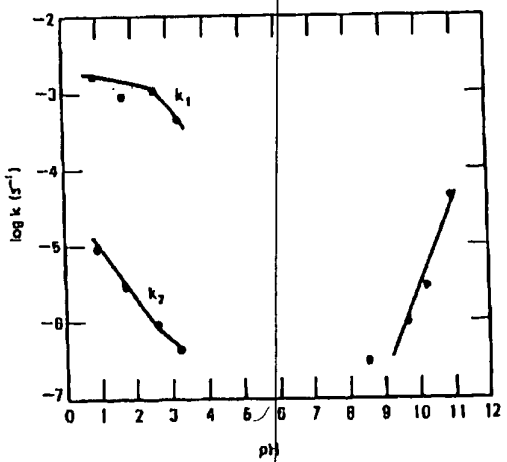


FIGURE 1. Diazepam. pH-rate profile for hydrolysis at 80°C, μ = 1.0 (3).

Activation Energy

The apparent activation energies for the hydrolysis of diazepam in aqueous solution and in several mixed solvent systems are given in Table 1. Arrhenius plots for diazepam hydrolysis in aqueous solution under acidic and basic conditions are shown in Figure 2. The rate data shown for acid pH are for the more rapid reaction step, labeled k_1 in Figure 1. Extrapolated to room temperature, the rate constant for diazepam hydrolysis is $k = 1.41 \times 10^{-5}$ s^{-1} at pH 0.93 and $k = 2.95 \times .10^{-8}$ s^{-1} at pH 10.18, corresponding to a $t_{½}$ of 0.57 and 272 days, respectively. The Arrhenius plot for diazepam hydrolysis in the mixed solvent system C (Table 1), similar in composition to the commercial, parenteral formulation of diazepam, is given in Figure

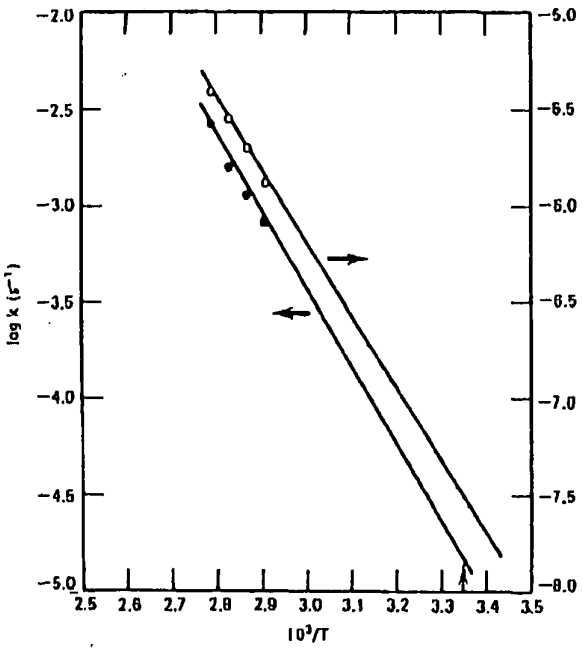


FIGURE 2. Diazepam. Arrhenius plots for diazepam hydrolysis at pH 0.93 (●, first reaction step) and pH 10.18 (○) (3). Vertical arrow indicates room-temperature points.

3. Extrapolated to room temperature, the rate constant is $k = 5.55 \times 10^{-11}\ s^{-1}$ (7,9).

FORMULATIONS AND COMBINATIONS

Degradation Reactions

The degradation of a number of benzodiazepine derivatives, including diazepam, in an injectable formulation, solvent system C in Table 1, has been studied by Carstensen et al. (7). While the benzophenone is the major decomposition product, small amounts of carbostyril and acridone derivatives are also observed at high temperatures; the formation rates of these at room temperature are 1/10 to 1/100 that of the benzophenone. Diazepam has a methyl group substituent in the 1-position and is hence more stable than many other 1,4-benzodiazepines (7).

TABLE 1. Apparent Activation Energies for Diazepam Degradation

Solvent System	Solvent Composition	pH	E_a (kcal/mol)	Ref.
A	Buffered aqueous solution	0.93	18.4[a] 22.7[b]	3
		10.18	17.2	
B	Polyethylene glycol 400[c] Ethanol Water	6.85 6.17	19.8 18.4	4
C	Propylene glycol 40% Ethanol 10% Benzyl alcohol 1.5% Water q.s.	--[d]	22.7	7
D	Polyethylene glycol 200 40% Ethanol 10% Benzyl alcohol 1.5% Benzoic acid 0.2% Sodium benzoate 9.8% Water q.s.	--[d]	23.3[e]	8

[a]For Eq. (2).
[b]For Eq. (4).
[c]Percent composition not specified.
[d]Not specified.
[e]Calculated from data given in reference.

Diazepam tablets are relatively stable; with tablet moisture content above 5%, decomposition was detected after storage at 60°C for 53 days (10). Storage of water-free diazepam suppositories for 12 weeks at 35°C resulted in 1.30% decomposition (10). Loss of diaze-

pam from solution via sorption to components and containers used in the parenteral administration of the drug has been reported by a number of authors (11-14).

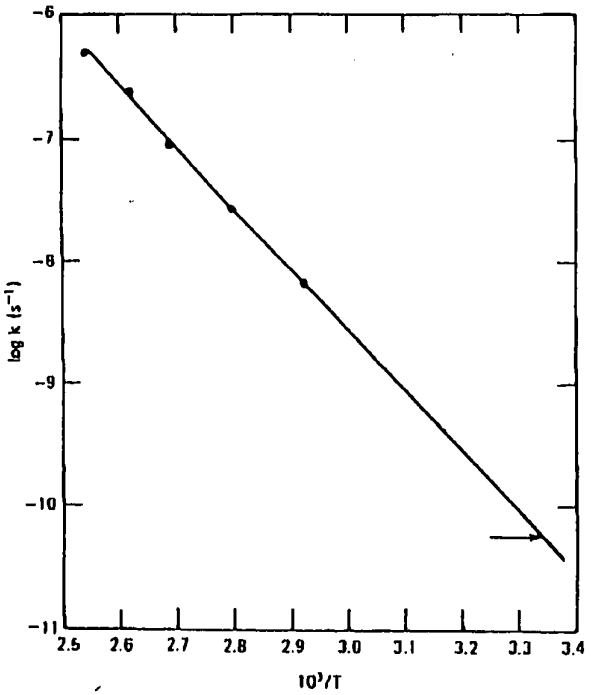


FIGURE 3. Diazepam. Arrhenius plot for diazepam degradation in a solvent system consisting of propylene glycol 40%, ethanol 10%, benzyl alcohol 1.5%, and water q.s. (9). Arrow indicates room-temperature point.

Stabilization Methods

Hydrolytic degradation of diazepam in solid dosage forms can be minimized by limiting moisture. Solutions of diazepam exhibit maximum stability near pH 5. Solutions of diazepam in mixed aqueous solvent systems, similar to that of the parenteral formulation, not only increase diazepam solubility but also result in enhanced stability relative to aqueous solutions.

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– M. R. Dobrinska
(Wisconsin)

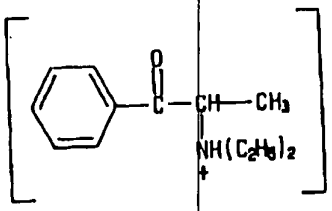
Diethylpropion Hydrochloride

GENERAL

Names

Diethylpropion hydrochloride.

Structure



$C_{13}H_{19}NO \cdot HCl$

mol. wt. 241.76

Forms Available

Diethylpropion hydrochloride.

Physical Properties

Melting point 175°C (decomposition). Solubility: 1 g in 0.6 mL water, 3 mL chloroform, or 3 mL alcohol; insoluble in ether. pK_a 8.7 (1-3).

Stability Summary

Diethylpropion undergoes hydrolysis in solution to form diethylamine and 1-phenyl-1,2-propanedione. Stability in aqueous solution is acceptable at pH values below 3.6. It also undergoes homolysis in the presence of light to give ethylamine, diethylamine, acetaldehyde, and propiophenone. The chemical degradation in solid dosage forms is minimized by preventing contact with moisture and light.

COPY PROVIDED FOR APPLN. NO. 10/750,940
(016914-039)

Patent Application
Attorney's Docket No. 016914-007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Thorsteinn LOFTSSON et al	Group Art Unit: 1623
Application No.: 09/250,185	Examiner: Lawrence E. Crane
Filed: February 16, 1999	Confirmation No.: 1768
For: HIGH-ENERGY CYCLODEXTRIN COMPLEXES	

FIRST DECLARATION OF MAR MASSON PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, MAR MASSON, declare as follows:

1. That I am a citizen of Iceland residing at Fjolnisvegur 1, 101 Reykjavik, Iceland.

2. That I graduated from the University of Iceland in 1987 with a B.S. in chemistry, from the Copenhagen University in 1990 with a Cand. Scient. Chemistry, from Japanese studies at the Tokyo Institute of Technology, Foreign Student Training Center in 1991, and from the Tokyo Institute of Technology in 1995 with a Ph.D. in Biotechnology.

3. That in 1995, I was employed by the University of Iceland, Faculty of Medicine, Department of Biochemistry, as a researcher; from 1995 to 1997, I was employed by the University of Iceland, Faculty of Medicine, Department of Pharmacy, in

research for Professor Thorsteinn Loftsson, Ph.D.; and in 1997, I was employed by the University of Iceland, Faculty of Medicine, in a post-doctoral position supervised by Professor Thorsteinn Loftsson, Ph.D. and Professor Einar Stefansson, M.D., Ph.D.

4. That from 1998 to the present, I have been employed by the University of Iceland, Faculty of Pharmacy, as Associate Professor in Medicinal Chemistry.

5. That I am one of the inventors of the above-referenced patent application.

6. That I am familiar with the prosecution history of this application, including the prior art relied upon by the Examiner.

7. That I am making this declaration to address the Examiner's belief that the ring opening may be inherently a part of the prior art.

8. That I have specifically considered the disclosure of the following: Loftsson and Brewster, *Pharm. Technol. Eur.*, 9(5), 26-34 (May, 1997), also referred to as Loftsson et al **AR**, and references cited therein and numbered **12, 14, 15, 16** and **18-20**, as well as Stella et al United States Patent No. 5,134,127, the teachings of which I summarize as follows:

(a) None of the prior art acknowledges the existence of the ring-opened form of benzodiazepines during complexation of benzodiazepines with any cyclodextrins or during administration of such complexes.

(b) None of the prior art mentions the contribution of the ring-opened form to the overall solubility of the benzodiazepines.

(c) No attempts were undertaken in the prior art to detect the ring-opened form of the benzodiazepines in cyclodextrin complexes.

(d) The references numbered **12, 15, 18, 19** and **20** are not relevant to the present invention because they do not relate to dissolving/complexing benzodiazepines with cyclodextrins.

(e) References **AR, 14** (Loftsson and Bodor, *Acta. Pharm. Nord.* 1, 185-194, 1989) and **16** (Loftsson, Gudmundsdottir, and Fridiriksdottir, *Drug. Devel. Ind. Pharm.*, 22, 401-405, 1996), copies of which are appended, present some data for the solubility of medazepam and prazepam in hydroxypropyl-β-cyclodextrin solutions.

(f) In the prior art examples of complexation, the concentrations of medazepam and prazepam were measured at pH 3.5, 3.8 and above this pH; in one instance (reference **16**, Figure 2), data for solubility of prazepam at pH 1 was presented.

9. That under my supervision and control, a study was carried out to investigate the presence and concentration of the ring-opened and ring-closed form of prazepam in 10% hydroxypropyl-β-cyclodextrin (HPβCD), at pH 1.0, 3.5, 4.0 and 5.0, as detailed below:

Procedure:

Preparation of solutions:

All chemicals used were of reagent or analytical grade. Solutions with pH 5.0, 4.0 and 3.5 were prepared in Theorell-Steinhagen buffer system. Solutions with pH 1.0 were prepared from 0.1 N HCl. Methanolic stock solution (20 mg/ml) of prazepam (Fabbrica Italiana sintetia, Italy) was prepared and used for creating a standard curve. Ten % (w/v) hydroxypropyl-β-cyclodextrin solutions (HPβCD, Encapsine from Wacker Chemie,

Germany) were prepared by weighing 0.5 g into 5 ml volumetric flasks. The aqueous buffer was added to dissolve the cyclodextrin, approximately 25 µl of the prazepam stock solutions were added by pipette (Gilson Inc., Wisconsin USA), the pH was adjusted, and the flasks were filled up to the mark. Then 1 ml aliquots of this solution were transferred to two 5 ml volumetric flasks. One flask was filled up to the mark with 10% (w/v) solution of HPβCD in the same buffer and the other flask was filled up to the mark with the mobile phase for the HPLC system. These solutions were then incubated for 1-2 days to achieve equilibrium between ring-open and ring-closed form in the system. The 10% HPβCD solutions were then analyzed by HPLC to determine the concentration of the ring-closed form of prazepam. The solutions that were diluted into the mobile phase were analyzed by HPLC to determine the total concentration of prazepam.

HPLC system:

Isocratic evaluation of the closed form of prazepam was done on HPLC (Agilent Technologies, 1100 Series, Germany) using Prodigy ODS (3u) 75 x 4.60 mm column (Phenomenex, USA). The UV detector was set to 230 nm, the flow rate was 1.5 ml/min. and MeOH:THF:H_2O = 85:1:14 was used as the mobile phase. [Loftsson, Gudmundsdottir, and Fridiriksdottir: The influence of water-soluble polymers and pH on Hydroxypropyl-β-cyclodextrin complexation of drugs. Drug Development and Industrial Pharmacy, 22(5), 401-405 (1996).] The standard curve was determined from the methanolic standards of prazepam. The samples were injected without any previous dilution.

Results:

Solutions consisting of prazepam diluted in the mobile phase or in the aqueous 10% HPβCD at the respective pH were assayed on HPLC as described. Prazepam can be partially in a ring-opened form in aqueous solutions, due to reversible hydrolysis of the imine bond in the ring system. Prazepam will be fully in ring-closed form when dissolved in organic solvent (e.g. the mobile phase). Two main peaks were observed when the aqueous solutions, which had been incubated overnight in buffer, were analyzed by HPLC (Appendix 1). The solvent front was observed after 1 min. and then the prazepam peak was observed with a 2.3 minutes retention time. The hydrophilic ring-opened form eluted with the solvent front. The effect of varying the organic solvent concentration in the mobile phase was investigated but the peak for the ring-opened form could not be fully separated from the solvent front. The peak for prazepam (ring-closed form) was larger in samples diluted and incubated in the mobile phase, and much smaller peaks were observed in the solvent front (Appendix 2.)

This investigation showed that the relative concentration of ring-opened form was highest at pH 1.0 (26.3% of the total concentration in the ring-opened form) and lowest at pH 5.0 (2.0% of the total concentration in the ring-open form).

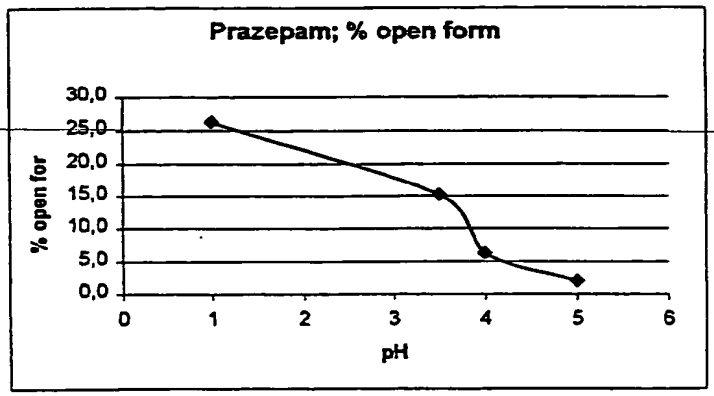


Figure 1:

The % of open form of Prazepam in solutions with different pH.

Buffer system:	pH	µg/ml total (dil. in mobile phase)	µg/ml closed, (dil. in 10% HPβCD)	% Closed	% Open
0.1N HCl	1.0	9.9	7.3	73.7	26.3
Theorell-Steinhagen	3.5	13.8	11.7	84.8	15.2
Theorell-Steinhagen	4.0	17.5	16.4	93.7	6.3
Theorell-Steinhagen	5.0	14.8	14.5	98.0	2.0

Table 1:

The data for % ring-opened and closed form of prazepam at different pH.

10. That the data shown above indicated that some ring-opened form of prazepam was obtained in hydroxypropyl-β-cyclodextrin when complexed according to the prior art, with a maximum amount (26.3%) of ring-opened form being obtained at pH 1.0; and that much lower amounts of ring-opened form were obtained at the higher acidic pH levels much more typically used in the prior art.

11. That this test shows that, although the art never detected or suggested the presence of the opened-ring form, some opened-ring form was produced repeating the prior art procedure for prazepam.

12. That I attempted to obtain medazepam to conduct a similar experiment with it in view of the prior art report of it at pH 3.8 in HPβCD, but was unable to obtain the drug.

13. That despite the inability to directly test medazepam, I note that the data shown in Table 3 of the present application, which was obtained in similar manner to that obtained above, together with the data given above for prazepam/HPβCD, clearly show that at pH 4, much less than 50% opened-form was obtained for the various benzodiazepines/cyclodextrins tested there, which leads me to conclude that less than 50% of medazepam would have been in opened form at pH 3.8 in hydroxypropyl-β-cyclodextrin.

14. Therefore, I believe that not only did the art fail to recognize or suggest the presence of the opened-ring form of benzodiazepines in cyclodextrin complexation, but the art did not even unknowingly produce the opened-ring form in an amount approaching the 50% level specified in applicants' claims.

15. That I further believe that because the ordinary skilled worker is not taught by the prior art anything about the existence of the opened-ring form in the complexation medium, he would not have any reason to think that he could enhance complexation of benzodiazepines with cyclodextrins by complexing the benzodiazepine with the cyclodextrin in an aqueous medium at a pH level below about 5 and allowing the resultant medium to equilibrate for sufficient time to effect chemically reversible ring-opening of 50% or more by weight of the benzodiazepine.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 23/4/2003

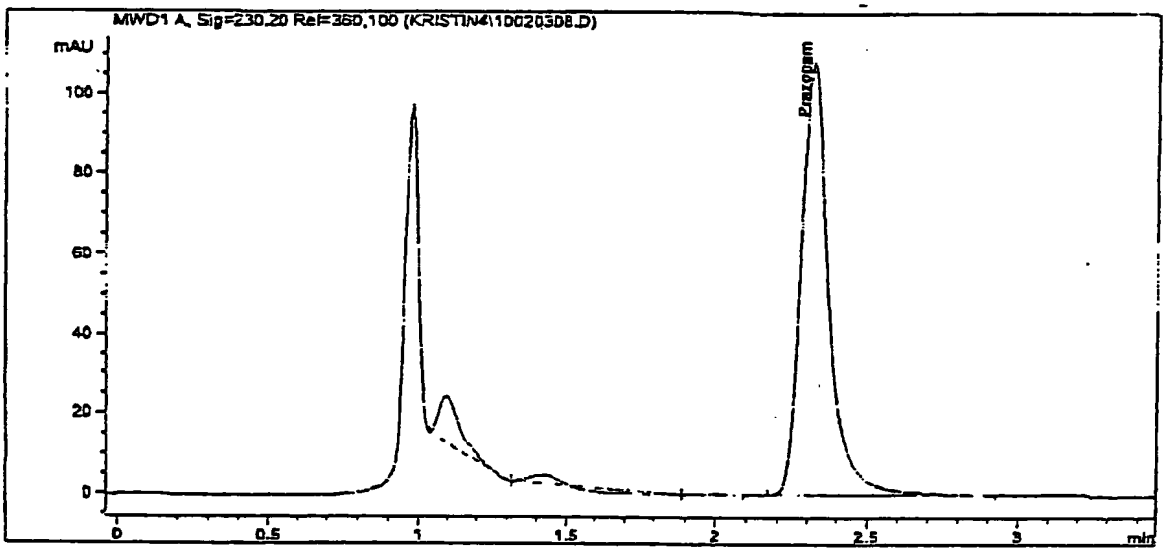
MAR MASSON

Appendix 1: Chromatogram for pH 1.0 diluted in the aqueous solution containing 10% HPβCD at pH 1.0 (for detection of the closed form at pH 1.0).

Sample Name: pH 1.0 c
Data file : C:\HPCHEM\1\DATA\KRISTIN4\10020308.D

Injection Date	: Mon, 10. Feb. 2003	Location	: Vial 8
Operator	: Kristin	Inj. No.	: 1
Method	: PRAZEPKT.M	Inj. Vol.	: 20 µl
Column	: Phenomex Prodigy 3u ODS(3) 100A		
Method info	: maeling a synum a,b,c mism pH		

Mobile phase :

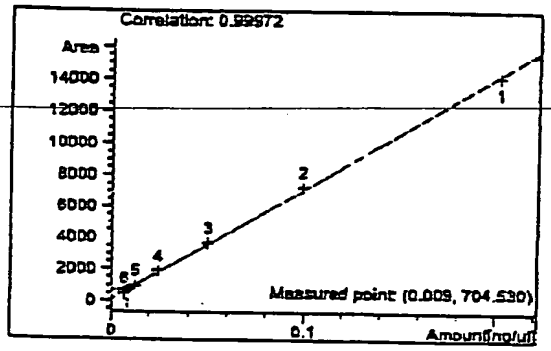


Calib. Data Modified :Fri, 21. Mar. 2003,10:04:47 am
Multiplier :1.000000
Dilution :1.000000
Uncalibrated Peaks :not reported

Signal Description :MWD1 A, Sig=230,20 Ref=360,100

RT [min]	Height	Width	Symmetry	Area [mAU*s]	Amount [%]
1.09289	11.66515	0.07723	0.48187	59.22594	0.00000
1.42227	2.23321	0.03583	0.61763	1.49842	0.00000
1.93514	0.10694	0.05855	0.50435	0.40834	0.00000
2.31031	108.64189	0.09766	0.64279	704.52960	0.00859
Totals:					

Appendix 1, continued



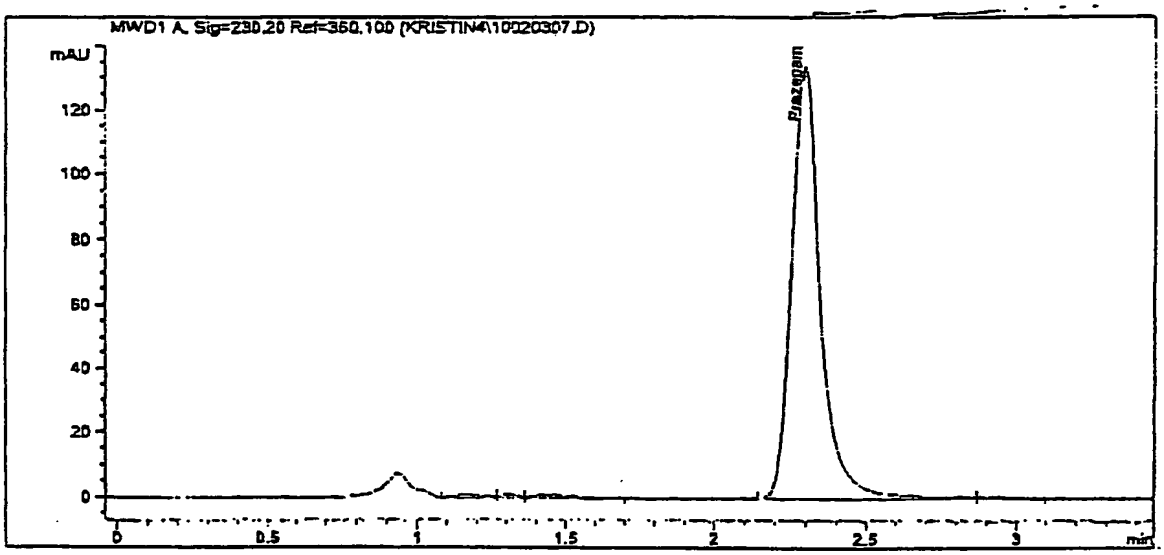
Cal. Curve Formula: Area = 70184.8562*Amt +101.90691
Cal. Curve Type : Area
Calc. based on : Linear

Appendix 2: Chromatogram for pH 1.0 diluted in the mobile phase for detection of the total concentration of prazepam.

Sample Name: pH 1.0 b
Data file : C:\HPCHEM\1\DATA\KRISTIN4\10020307.D

Injection Date	: Mon, 10. Feb. 2003	Location	: Vial 7
Operator	: Kristin	Inj. No.	: 1
Method	: PRAZEPKT.M	Inj. Vol.	: 20 µl
Column	: Phenomex Prodigy 3u ODS(3) 100A		
Method info	: maeling a synum a,b,c mism pH		

Mobile phase :



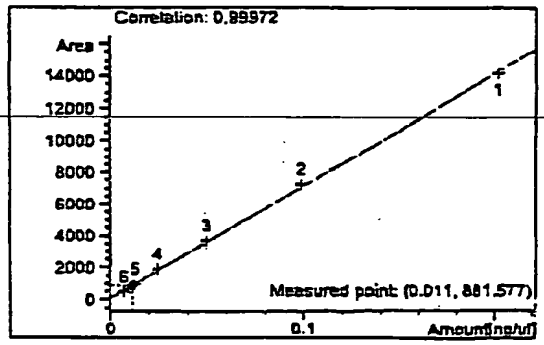
Calib. Data Modified :Fri, 21. Mar. 2003,10:05:50 am
Multiplier :1.000000
Dilution :1.000000
Uncalibrated Peaks :not reported

Signal Description :MWD1 A, Sig=230,20 Ref=360,100

RT [min]	Height	Width	Symmetry	Area [mAU*s]	Amount [%]
1.16271	0.96118	0.12055	0.55696	7.95465	0.00000
1.31127	1.07077	0.05715	1.04314	4.14368	0.00000
1.44111	1.02064	0.12433	0.58984	8.42722	0.00000
2.28806	133.88298	0.09882	0.66406	881.57733	0.01111
2.90565	0.30637	0.09257	0.42978	2.01081	0.00000

Totals:

Appendix 2, continued



Cal. Curve Formula: Area = 70184.8562*Amt +101.80693
Cal. Curve Type : Area
Calc. based on : Linear

COPY PROVIDED FOR APPLN. NO. 10/750,940
(016914-039)

Patent Application
Attorney's Docket No. 016914-007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Thorsteinn LOFTSSON et al	Group Art Unit: 1623
Application No.: 09/250,185	Examiner: Lawrence E. Crane
Filed: February 16, 1999	Confirmation No.: 1768
For: HIGH-ENERGY CYCLODEXTRIN COMPLEXES	

FIRST DECLARATION OF THORSTEINN LOFTSSON PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, THORSTEINN LOFTSSON, declare as follows:

1. That I am a citizen of Iceland residing at Sorlaskjol 44, IS-107 Reykjavik, Iceland.

2. That I received my M.S. Pharm. degree from the Royal Danish School of Pharmacy in Copenhagen in 1975, and my M.S. and Ph.D. degrees in 1978 and 1979, respectively, from the Department of Pharmaceutical Chemistry at the University of Kansas in Lawrence, Kansas.

3. That I have authored or co-authored over 160 papers in peer-reviewed journals, 10 book chapters, 20 patents and patent applications and about 180 abstracts.

4. That in 1998 I became a Fellow of the American Association of Pharmaceutical Sciences.

5. That I have received the following honors, awards and recognition: ~~Fulbright Fellowship (1976-1979), NATO Awards in 1978 and 1980, The Nagai~~ Foundation Tokyo, International Scholarship in 1998 and the 1992 Faculty Research Award, Faculty of Medicine, University of Iceland.

6. That I have served as a board member and a referee for numerous scientific journals and am currently a member of the editorial board of *International Journal of Pharmaceutics*, *Die Pharmazie* and *STP Pharma Sciences*.

7. That since 1979 I have been employed by the University of Iceland, Department of Pharmacy, Reykjavik, Iceland, as an Assistant Professor from 1979 to 1983, as an Associate Professor from 1983 to 1985, and as Professor of Physical Pharmacy from 1986 to the present.

8. That I have also been an Adjunct Associate Professor in the Department of Medicinal Chemistry, College of Pharmacy, University of Florida, Gainesville, Florida, from 1980 to 1987, and an Adjunct Professor, Center for Drug Discovery, College of Pharmacy, University of Florida, Gainesville, Florida, from 1987 to 1996.

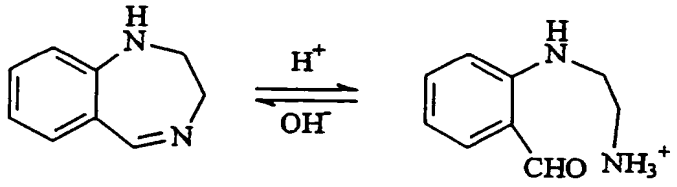
9. That I am one of the inventors of the above-referenced patent application.

10. That I am making this declaration to address the Examiner's apparent confusion between ionization and ring-opening.

11. That all experiments referred to below were conducted under my supervision and control.

12. That I submit the following comments and experimental data to clarify the roles of ionization and ring-opening in cyclodextrin complexation of benzodiazepines:

13. In aqueous solutions, some drugs can exist in more than one structural form, e.g. equilibrium isomers or ionization stages. Although the individual forms are in equilibrium with each other, and thus not totally independent of each other, the overall aqueous solubility (or apparent S_o) of a drug can be enhanced through formation of such multiple structural forms. Cyclic imines, such as 2,3-dihydro-1*H*-1,4-benzodiazepine, are known to undergo reversible and pH-dependent ring-opening through formation of aldehyde or ketone and a primary amine:



Under certain conditions opened and closed forms are both present in aqueous solutions. Coexistence of such forms increases the apparent solubility of the benzodiazepine. Often, the ring-opened form is an intermediate which is formed during benzodiazepine degradation in aqueous solutions but in some cases, e.g. in the case of alprazolam, midazolam and triazolam, this form is chemically stable and can contribute to the overall aqueous solubility of the drug (Cho et al., "Kinetics and equilibrium of the reversible alprazolam ring-opening reaction", *J. Pharm. Sci.* 72, 356-362, 1983); Kanto, "Midazolam: the first water-soluble benzodiazepine. Pharmacology, pharmacokinetics and efficacy in insomnia and anaesthesia", *Pharmacotherapy* 5, 138-155, 1985; Kurono et al., "The behavior of 1,4-

benzodiazepine drugs in aqueous media. II. Kinetics and mechanism of the acid-base equilibrium reaction of oxazolam", *Chem. Pharm. Bull.* 33, 1633-1640, 1985; Kuwayama et al., "The behavior of 1,4-benzodiazepine drugs in acidic media. V. Kinetics of hydrolysis of haloxazolam in aqueous solution", *Chem. Pharm. Bull.* 34, 320-326,1986; Sbarbati Nudelman and de Waisbaum, "Acid hydrolysis of diazepam. Kinetic study of the reactions of 2-(*N*-methylamino)-5-chlorobenzophenone, with HCl in MeOH-H_2O", *J. Pharm. Sci.* 84, 998-1004, 1995). For example, in the commercial aqueous intravenous (i.v.) solution of midazolam (Dormicum®, Hoffmann-La Roche, Switzerland) the drug is 15 to 20% in the ring-open form and the pH is approximately 3.3 (Gerecke, "Chemical structure and properties of midazolam compared with other benzodiazepines", *Br. J. Clin. Pharmacol.* 16 (Suppl. 1), 11S-16S, 1983). In addition, both forms, i.e. the ring-opened and the ring-closed midazolam, can exist in several different ionization forms. In the aqueous i.v. solution, the ring-opened form of midazolam can be characterized as a midazolam prodrug since the ring is completely closed when the pH is elevated to 7.4. Previously my coworkers and I have shown that low complexation efficiency can hamper the usage of cyclodextrins in certain pharmaceutical formulations and that both drug ionization and water-soluble polymers can enhance the complexation efficiency (Loftsson, "Increasing the cyclodextrin complexation of drugs and drug bioavailability through addition of water-soluble polymers", *Pharmazie* 53, 733-740, 1998; Loftsson et al., "Methods to enhance the complexation efficiency of cyclodextrins", *STP Pharm. Sci.* 9, 237-242, 1999). Ionization of a drug molecule increases the apparent S_o and addition of a water-soluble polymer to the complexation media increases K_c.

14. Several investigators have attempted to use the commercially available aqueous i.v. solution for intranasal (i.n.) administration of midazolam (Björkman et al., "Pharmacokinetics of midazolam given as an intranasal spray to adult surgical patients", *Br. J. Anaesthiol.* 79, 575-580, 1997; Burstein et al., "Pharmacokinetics and pharmacodynamics of midazolam after intranasal administration", *J. Clin. Pharmacol.* 37, 711-718, 1997). The midazolam concentration in this solution is only 5 mg/ml. Thus, relatively large amounts of the acidic i.v. solution have to be sprayed into the nose in order to induce sedation and anxiolysis. Subsequently midazolam is only partly absorbed from the nasal cavity and partly from the gastrointestinal tract after swallowing. The midazolam bioavailability after i.n. administration of the i.v. solution is frequently about 50% (Burstein et al., 1997, id.). To reduce spilling and swallowing of the i.v. solution after i.n. administration, and to improve the bioavailability, the dosage has to be sprayed in small aliquots into the nasal cavity (Björkman et al., 1997, id.). However, i.n. administration of the acidic i.v. solution can cause severe irritation in the nasal cavity.

15. The purpose of the present study was to investigate the effects of the reversible ring-opening of the diazepine ring and ionization on the cyclodextrin complexation of benzodiazepines as well as formulation and testing of physiologically acceptable aqueous midazolam nasal spray solution.

1. Materials and methods

1.1 Materials

Midazolam base was purchased from Sifa (Shannon, Ireland), and alprazolam and triazolam from Sigma (St. Louis, MO). Sulfobutylether-β-cyclodextrin sodium salt with molar substitution of 6.2 (Capisol®, SBEβCD) was donated by CyDex (Kansas City, KS).

Randomly methylated β-cyclodextrin with degree of substitution (DS) of 1.8 (RMβCD) and 2-hydroxypropyl-β-cyclodextrin with DS of 0.6 (HPβCD) were donated by Wacker-Chemie (Burghausen, Germany). Hydroxypropyl methylcellulose 4000 (HPMC) was purchased from Mecobenzon (Denmark). All other chemicals used were of pharmaceutical or special analytical grade.

1.2 Solubility studies

An excess amount of the drug to be tested was added to water or aqueous Teorell-Stenhagen buffer system (Bates and Paabo, "Measurement of pH" in: Fasman (ed.), *Practical Handbook of Biochemistry and Molecular Biology*, CRC Press, Boca Raton, FL, 535-550, 1989), containing various amounts of the different cyclodextrins with or without a polymer. The suspension formed was heated in an autoclave in a sealed container to 130°C for at least 30 min. After cooling to room temperature (22-23°C), a small amount of solid drug was added to the container to promote precipitation. Then the suspension was allowed to equilibrate for at least 3 days at room temperature, protected from light. After equilibration was attained, an aliquot of the suspension was filtered through a 0.45-μm membrane filter (cellulose acetate from Schleicher & Schuell, Germany), diluted with the HPLC mobile phase and, after equilibration, analyzed by HPLC. The pH values reported were determined at room temperature at the end of the equilibration period.

The effect of pH on the stability constant (K_c) of the drug/cyclodextrin (1:1) complex was determined as previously described (Loftsson and Petersen, "Cyclodextrin solubilization of ETH-615, a zwitterionic drug", *Drug Dev. Ind. Pharm.* 24, 365-370, 1998). Briefly, the drug solubility was determined in aqueous nasal formulation containing

from 0 to 14% (w/v) cyclodextrin. The composition of the nasal formulation was as follows: benzalkonium chloride (0.02% w/v), EDTA (sodium edetate) (0.1% w/v), HPMC (0.1% w/v), phosphoric acid (0.43% v/v) and aqueous sodium hydroxide solution (for pH adjustment) in water. As before, the exact pH of each solution was determined at the end of the equilibration period. Differences in pH were corrected by drawing the pH-solubility profiles at each cyclodextrin concentration and determining the solubilities of the drug from these profiles at selected pH values. The values obtained were used to draw the phase-solubility diagrams, all of which were linear. Finally, K_c was calculated from the equation (Higuchi and Connors, "Phase-solubility techniques", *Adv. Anal. Chem. Instrum.* 4, 117-212, 1965):

$$K_c = \frac{\text{Slope}}{S_o(1 - \text{Slope})}$$

where K_c is the stability constant of the drug-cyclodextrin (1:1) complex, slope is the calculated slope of the linear phase-solubility diagram and S_o is the apparent intrinsic solubility of the free drug determined in the aqueous complexation media, at appropriate pH, when no cyclodextrin or polymer was present.

1.3 Quantitative determinations

The quantitative determination of drugs was carried out on a high performance liquid chromatographic (HPLC) component system consisting of ConstaMetric 3200 isocratic solvent delivery system operated at 1.50 ml/min, a Merck-Hitachi AS4000 autosampler, a Luna C_{18} 5 μm (4.6 x 150 mm) column, a Spectro Monitor 3200 UV/VIS variable-wavelength detector and a Merck-Hitachi D-2500 Chromato-Integrator. The

mobile phase for alprazolam and triazolam consisted of methanol and water (68.32). The pH of the mobile phase was adjusted to 2.7 by addition of trifluoroacetic acid. The flow rate was 1.0 ml/min and the detector was operated at 254 nm. For alprazolam, the retention was 2.8 min for the ring-opened form and 4.7 min for the ring-closed form. For triazolam the retention was 2.3 min for the ring-opened form and 3.9 min for the ring-closed form. The mobile phase for midazolam consisted of pH 7.2 aqueous 0.004 M phosphate buffer, acetonitrile and triethylamine (55:45:0.1). The flow rate was 1.5 ml/min and the detector was operated at 240 nm. The retention time was 2.6 min for the ring-opened form and 4.2 min for the ring-closed form.

When the fraction of ring-opened form was determined, the concentration of the closed form was determined right after dissolving the benzodiazepine in the aqueous buffer solution, containing either no cyclodextrin or 10% (w/v) cyclodextrin, and again 24 h later (i.e. after equilibration at 23°C). Preliminary experiments had shown that equilibrium between the closed and the opened form was attained within 3 h at 23°C and that no degradation of either the ring-opened or the ring-closed form occurred during the 24-h experiment.

1.4 Kinetic studies in the aqueous buffer solutions

A stock solution (1.0×10^{-3} M) of the drug to be tested was prepared in a 0.1 M aqueous hydrochloric acid solution (pH 1). This solution was equilibrated in a 37°C water bath for 3 h. This was to ensure that mainly the ring-opened form was present in the stock solution. Cyclodextrin, ethanol or dimethyl sulfoxide (DMSO) was dissolved in, or mixed with, pH 7.5 aqueous 0.5 M tris(hydroxymethyl)aminomethane (Tris) buffer solution and

the solution equilibrated at 37°C. At time zero, 30 μl of the stock solution was added to 1.5 ml of the buffer solution, mixed for a couple of seconds on a vortex mixer, and placed again in the 37°C water bath. At various time points samples were withdrawn from the reaction media and injected into a HPLC system (see Section 1.3). Both the ring-opened and the ring-closed forms could be detected by HPLC and the disappearance of the ring-opened form was proportional to the appearance of the ring-closed form. The first-order rate constants (k_{obs}) for the disappearance of the ring-opened form was calculated by linear regression of the natural logarithm of the peak height versus time plots.

1.5 Kinetic studies in human serum

The rate constant for the ring-closing reaction was determined in serum. The previously described (Section 1.4) stock solution of the drug (15μl) was added to 1485μl of serum which had previously been equilibrated at 37°C. After thorough mixing on a vortex mixer for a couple of seconds the solution was placed in a 37°C water bath. Sample (100 μl) was withdrawn from the solution at various time points and mixed with 900 μl of ice cold methanol and the solution sonicated for 1 min. Then the solution was centrifuged and the clear supernatant analyzed by HPLC.

1.6 Formulation of the aqueous nasal spray solution

The phase solubility of midazolam was determined in a medium which closely resembled the aqueous nasal spray vehicle, i.e. 7 - 13% (w/v) SBEβCD, 0.10% (w/v) HPMC, 0.02% (w/v) benzalkonium chloride, 0.10% (w/v) EDTA and 0.43% (v/v) concentrated phosphoric acid. Excess midazolam was added to this medium and the pH adjusted to 4.35 with concentrated aqueous sodium hydroxide solution, both before and

after heating in an autoclave (121°C for 40 min). Then the samples were allowed to equilibrate for at least 4 days at room temperature and analyzed as before (Section 1.2). The exact composition of the nasal spray was based on this study. The viscosity of the nasal spray was determined with a Brookfield viscometer (UK) fitted with a ULA-DIN spindle and an UL sample holder with water-circulation jacket (25°C). The osmolarity of the nasal spray was measured by the freezing point depression method using a Knauer Osmometer Automatic (Netherlands). The buffer capacity of the nasal spray was estimated by the titration method using an aqueous 0.1 N sodium hydroxide solution. The preliminary evaluation of the chemical stability of midazolam in the nasal formulation was performed by determining the midazolam concentration after successive heating cycles in an autoclave (Midmark M7 SpeedClave). Each heating cycle consisted of heating to 121°C, maintaining this temperature for 20 min, and cooling to room temperature. The midazolam concentration was determined after each heating cycle. The total number of heating cycles was six. Finally the midazolam nasal spray was stored at room temperature (22 - 23°C) and samples collected at 0, 3, 4 and 12 months and analyzed.

2. **Theoretical background**

All the benzodiazepine drugs studied, i.e. alprazolam, midazolam and triazolam, contained 2,3-dihydro-1*H*-1,4-benzodiazepine structure (Fig. 1).

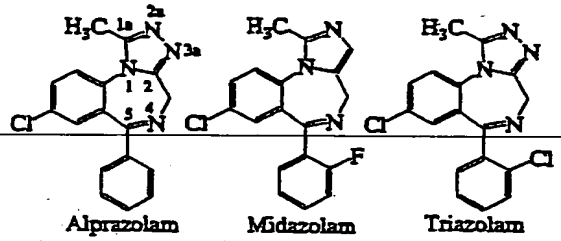


Fig. 1. The chemical structures of the benzodiazepine bases studied.

Alprazolam and triazolam have a 1*H*-1,2,4-triazole ring fused on the 1,2-carbon-nitrogen bond of the diazepine nucleus (i.e. a triazolo [4,3-*a*][1,4]benzodiazepine structure), whereas midazolam has an imidazole ring fused on the 1,2-carbon-nitrogen bond (i.e. an imidazo [1,5-*a*][1,4]benzodiazepine structure). Imidazole is relatively basic (pK_a 6.9) compared to 1*H*-1,2,4-triazole. Thus, in midazolam the protonated nitrogen in position 2 on the imideazole ring (i.e. N-2a) has a pK_a of 6.15 whereas in alprazolam and triazolam the protonated N-2a on the triazole ring has $pK_a \leq 1.5$ (Walser et al., "Quinazolines and 1,4-benzodiazepines. Synthesis and reactions of imidazo [1,5-a][1,4]benzodiazepines", *J. Org. Chem.* 43, 936-944, 1978). In the diazepine nucleus the protonated nitrogen in position 4 (i.e. N-4) has been estimated to be about 2.4 (Cho et al., 1983, id.). In aqueous solutions the benzodiazepines undergo a reversible and pH-dependent ring-opening reaction (Fig. 2) (Han et al, "Kinetics and mechanisms of hydrolysis of 1,4-benzodiazepines I: chlodiazepoxide and demoxepam", *J. Pharm. Sci.* 65, 1198-1204, 1976; Han et al., "Kinetics and mechanisms of hydrolysis of 1,4-benzodiazepines II: oxazepam and

diazepam", *J. Pharm. Sci.* 66, 573-577, 1977a; and Han et al, "Kinetics and mechanisms of hydrolysis of 1,4-benzodiazepines III: nitrazepam, *J. Pharm. Sci.* 66, 795-798, 1977b; Cho et al., 1983, id.). The pK_a of the primary nitrogen formed has been estimated to be about 7.0 (Cho et al., 1983, id.). There are some indications that the ring-opening should be pH-independent (Cho et al., 1983, id.) in which case the ring-opening rate constant (k_1) can be described by

$$k_1 = k_{H_2O} f_{HB+} \qquad (1)$$

where k_{H_2O} is the pH-independent rate constant and f_{HB+} is the fraction of benzodiazepine which is protonated in position N-4. However, Eq. (1) is kinetically equivalent to Eq. (2)

$$k_1 = k_H[H^+] f_B \qquad (2)$$

$$f_{HB+} = 1 - f_B = \frac{[H^+]}{[H^+] + K_a} \qquad (3)$$

where k_H is the specific acid catalysis rate constant for the ring-opening reaction, $[H^+]$ is the hydronium concentration and f_B is the fraction of benzodiazepine which is not protonated in position N-4. Comparable equations can be obtained for the ring-closing rate constant (k_{-1}). Under normal conditions the ring-opened forms of alprazolam, midazolam and triazolam are chemically stable in aqueous solutions.

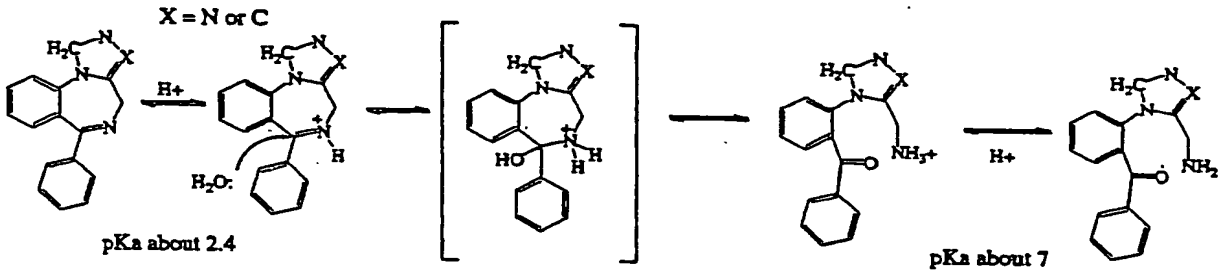


Fig. 2. The ring-opening reaction of benzodiazepines.

Table 1
The apparent equilibrium constant between the closed and open forms of the benzodiazepines:

$$K_{eq} = \frac{[open]_{Total}}{[closed]_{Total}}$$

where $[open]_{Total}$ is the total concentration of benzodiazepine which is in the ring-open form and $[closed]_{Total}$ is the total concentration of benzodiazepine which is in the ring-closed form at 37°C

Cyclodextrin	pH	K_{eq}		
		Alprazolam	Midazolam	Triazolam
No cyclodextrin	1	20	50	20
	2	6	3	2
	4	0.3	0.1	<0.1
10% (w/v) HPβCD	1	100	100	20
	2	15	2	2
	4	0.3	<0.1	<0.1
10% (w/v) SBEβCD	1	100	100	50
	2	25	6	3
	4	0.8	0.1	<0.1
10% (w/v) RMβCD	1	100	50	20
	2	10	0.8	0.8
	4	0.1	0.1	<0.1

3. Results and discussion

3.1 Solubilization

The aqueous solubility of benzodiazepines is a function of both the ionization of the drug molecule and the ring-opening of the diazepine ring. The ring-opening of the benzodiazepine ring is pH-dependent and fully reversible (Fig. 2). The observed equilibrium constant (K_{eq}) between the total concentration of the opened and closed forms is pH-dependent, strongly favoring the closed form at pH above 4, but the opened form at pH below 2 (Table 1). In general, the cyclodextrins appear to stabilize the ring-open forms (i.e. OH^+ and OH_2^{2+}) resulting in an increased K_{eq} value at low pH. The data presented in Fig. 3 are based on solubility studies, quantitative determination of the total amounts of the ring-opened and ring-closed benzodiazepine forms, and the observed pK_a values of the different benzodiazepine forms in pure aqueous solution.

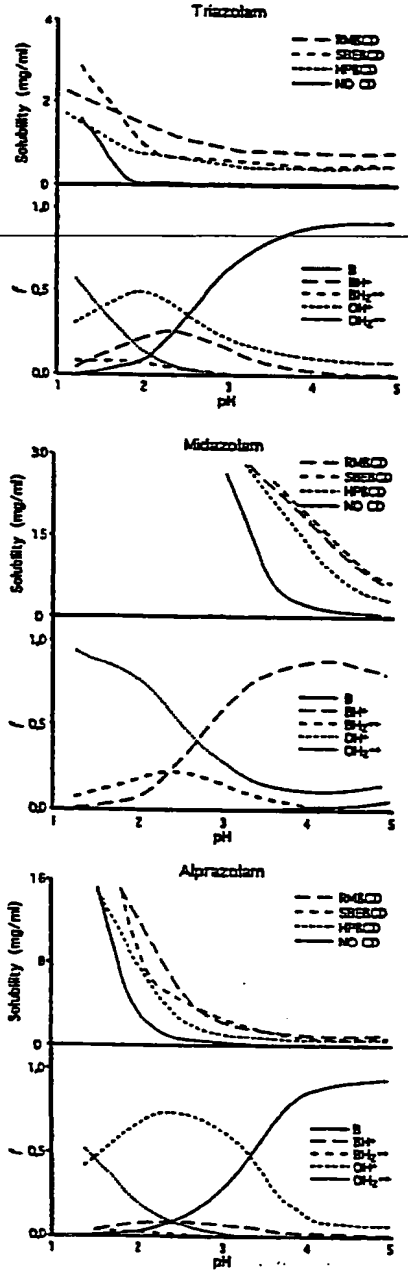


Fig. 3. The effects of cyclodextrins, ionization and ring-opening on the aqueous solubility of benzodiazepines at room temperature (22–23°C). The cyclodextrin concentration was 10% (w/v). f, mol fraction; B, benzodiazepine base (ring-closed form); BH^+, monoprotonised benzodiazepine; BH_2^{2+}, diprotonised benzodiazepine; OH^+, monoprotonised ring-open form; OH_2^{2+}, diprotonised ring-open form.

From Fig. 3, it is possible to estimate the contribution of each species (i.e. different ionization forms of both the ring-opened and ring-closed forms) to the overall benzodiazepine solubility in aqueous solutions. For example, it is clear that the monoprotonized (BH^+) and diprotonized (BH_2^{2+}) ring-closed forms, as well as the monoprotonized ring-opened forms (OH^+), have an insignificant effect on the overall aqueous solubility of the three benzodiazepines studied. Only when the diprotonized ring-opened forms (OH_2^{2+}) emerge do we observe a notable increase in aqueous solubility. Furthermore, it is apparent that the uncharged cyclodextrins (i.e. RMβCD and HPβCD) interact less strongly with OH^+ and OH_2^{2+} than with the uncharged ring-closed form B, BH^+ or BH_2^{2+} (Fig. 3). However, the negatively charged SBEβCD interacts somewhat more strongly than the uncharged cyclodextrins with OH_2^{2+} resulting in enhanced solubilization at low pH. The pK_a values of midazolam are about 2.4 (N-4) and 6.15 (N-2a) while those of alprazolam and triazolam are about 1.5 (N-2a) and 2.4 (N-4). Thus, the main reason for greater aqueous solubility of midazolam with decreasing pH, compared to the other two benzodiazepines studied, is the early appearance of the protonized forms, especially the diprotonized OH_2^{2+} form.

Cyclodextrins are able to form 1:1 complexes with the protonized forms and, thus, they are able to solubilize the positively charged ring-opened and ring-closed forms (Figs. 3 and 4). However, the stability constants of these complexes are somewhat lower than those of comparable uncharged species. It is possible to increase the complexation efficacy by adding a small amount of a water-soluble polymer to the aqueous complexation media and heating (Loftsson, 1998, id.; Loftsson et al., 1999, id.). For midazolam, SBEβCD was the

best solubilizer of the three cyclodextrins tested and addition of 0.10% (w/v) hydroxypropyl methylcellulose (HPMC) and heating in an autoclave at 121°C for 20 - 40 min enhanced its solubilizing effect (Fig. 4). The value of the stability constant of the midazolam/SBEβCD (1:1) complex was determined to be 700 M^{-1} at pH 4.8 but 425 M^{-1} at pH 4.0.

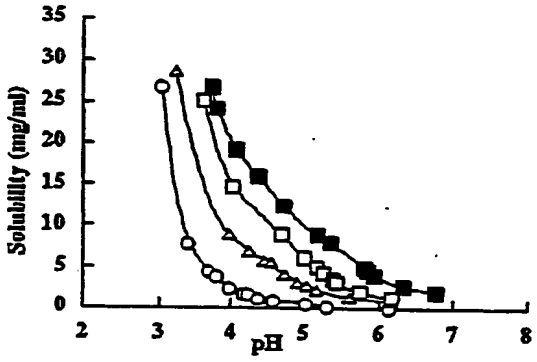


Fig. 4. The effects of pH and cyclodextrins on the solubility of midazolam in aqueous Teorell–Stenhagen buffer system. No cyclodextrin present (○); 10% (w/v) HPβCD (△); 10% (w/v) SBEβCD (□); 10% (w/v) SBEβCD and 0.10% (w/v) HPMC (■).

3.2 Kinetic studies

Equilibrium between the ring-opened and ring-closed forms is reached within a few minutes upon dissolution of the benzodiazepine in aqueous media. The equilibrium constants are pH-dependent favoring the ring-opened forms at low pH and the ring-closed forms at physiological pH (Table 1). It is believed that only the ring-closed forms of the benzodiazepines re pharmacologically active. Thus, it is of interest to determine how fast the ring closes under physiological conditions. The half-life of the first-order rate constant

was determined in aqueous 0.5 M Tris buffer solution at pH 7.5 and 37°C. For alprazolam the half-life in pure aqueous buffer solution was determined to be 5.3 min, 3.9 min for midazolam and 53 min for triazolam. Addition of cyclodextrins to the aqueous reaction medium increased the half-life of the ring-closing reaction (Table 2). This effect of cyclodextrins on the half-life is in agreement with the observation that cyclodextrins stabilize the ring-opened forms (i.e. OH^+ and OH_2^{2+}). Organic solvents such as ethanol and dimethylsulfoxide reduce the complexation by competing with the benzodiazepines for a space in the cyclodextrin cavity and, thus, reducing the effects of cyclodextirns. However, when no cyclodextrin was present in the reaction medium both ethanol and dimethylsulfoxide increased the half-life for the ring-closure of alprazolam and midazolam. In the case of triazolam the effects were much less pronounced.

In freshly collected human serum the half-life of the first-order rate constant for the ring-closing reaction was estimated to be less than 2 min for both alprazolam and midazolam (in vitro at 37°C). For triazolam the half-life was somewhat higher but still very short. Thus, it can be assumed that ring-opened forms of the benzodiazepines close very rapidly upon absorption into the systemic circulation.

In the nasal cavity lipophilic molecules will compete with the drug molecules for a space in the cyclodextrin cavity in much the same way as ethanol and DMSO molecules do in the in vitro study. Thus, administration of the ring-opened form of benzodiazepines in a cyclodextrin-containing nasal spray solution should not have any effect on their pharmacological effect. That is beside enhancing aqueous solubility and delivery of the drug molecule through the biological membrane. However, excess cyclodextrin can

decrease the drug bioavailability in the nasal spray solution (Masson et al., "Cyclodextrins as permeation enhancers: some theoretical evaluations and *in vitro* testing", *J. Controlled Release* 59, 107-118, 1999). It is therefore important to use just enough cyclodextrin to solubilize the drug in the aqueous nasal spray solution.

Table 2
The effects of cyclodextrins and organic cosolvents on the half-life for the rate of ring-closure in aqueous 0.5 M Tris buffer solution at pH 7.5 and 37.0°C

Cyclodextrin 10% (w/v)	Organic cosolvent[a] % (v/v)	Half-life ratio[b]		
		Alprazolam[c]	Midazolam[c]	Triazolam[c]
No cyclodextrin	No cosolvent	1.0	1.0	1.0
	10% EtOH	1.1	1.4	1.0
	50% EtOH	1.8	2.1	0.7
	10% DSMO	1.3	1.3	1.0
	50% DMSO	1.2	1.6	1.0
HPβCD	No cosolvent	4.2	6.5	2.0
	10% EtOH	2.5	4.6	1.4
	50% EtOH	1.8	2.7	1.3
	10% DSMO	2.9	5.3	1.7
	50% DMSO	1.5	2.1	1.2
SBEβCD	No cosolvent	4.2	13.7	2.3
	10% EtOH	2.5	5.4	1.6
	50% EtOH	2.2	2.8	1.4
	10% DSMO	2.7	8.0	1.8
	50% DMSO	1.4	2.1	1.2
RMβCD	No cosolvent	5.2	6.0	2.1
	10% EtOH	2.6	4.2	1.4
	50% EtOH	1.9	2.6	1.3
	10% DSMO	3.3	3.6	1.7
	50% DMSO	1.6	2.1	1.2

[a] EtOH, absolute ethanol, DMSO, dimethylsulfoxide.
[b] The half-life divided by the half-life in pure aqueous buffer solution (i.e. buffer solution containing neither cosolvent nor cyclodextrin).
[c] The half-life for formation of alprazolam, midazolam and triazolam in aqueous pH 7.5 buffer solution at 37.0°C was determined to be 5.3, 3.9 and 53 min, respectively.

3.3 Formulation of a midazolam nasal spray

The phase solubility of midazolam in the aqueous nasal spray vehicle shows that 12.33% (w/v) SBEβCD is required to dissolve 17 mg of midazolam in 1 ml of the vehicle (Fig. 5).

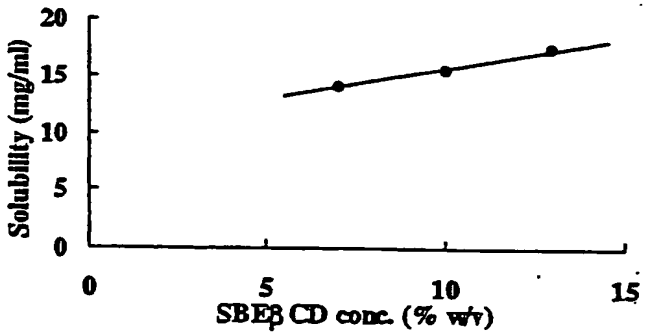


Fig. 5. The phase solubility of midazolam in the nasal spray vehicle at room temperature (22–23°C).

To ensure that no precipitation will be formed during storage, a small excess of SBEβCD is needed. Thus, the final formulation contained 14% (w/v) SBEβCD. The composition of the aqueous nasal formulation was as follows: midazolam (1.7% w/v), SBEβCD (14% w/v), HPMC (0.1% w/v), benzalkonium chloride (0.02% w/v), EDTA (0.1% w/v), concentrated phosphoric acid (0.43% v/v) and water (to 100% v/v). A concentrated aqueous sodium hydroxide solution was used to adjust the pH to 4.3. The nasal spray was prepared as follows. The solid components were weighed into a 100-ml volumetric flask. Phosphoric acid and most of the water was added and the solution stirred until all solid material had dissolved. Then the pH was adjusted to 4.35 with a concentrated sodium

hydroxide solution under stirring. Water was added to the mark and the aqueous solution heated in a sealed vessel contained in an autoclave (121°C for 40 min). After cooling, the solution was filtered through a sterile 0.45-μm membrane filter into amber glass vials under aseptic conditions.

The stability of midazolam in the nasal spray upon heating in an autoclave was investigated. The aqueous nasal spray solution was heated in sealed containers for up to six successive heating cycles. Each heating cycle consisting of heating to 121°C for 20 min and cooling to room temperature. The midazolam concentration in the solution was determined after each heating cycle by HPLC. No loss of midazolam could be detected in the nasal spray during heating in an autoclave. Further evaluation of the chemical stability of midazolam in the nasal spray solution was performed at room temperature. The solution was stored in several sealed containers in the dark, three containers were removed at various time points for up to 12 months and the midazolam concentration determined by HPLC. The degradation rate constant was estimated by linear regression of the natural logarithm of the peak height versus time plots. This yielded a half-life of approx. 350 months and an estimated 95% expiration limit (t_{95}) of over 2 years at room temperature. Thus, the ring-opened form of midazolam has adequate chemical stability in the aqueous nasal spray solution. The aqueous nasal spray solution showed Newtonian flow characteristics and its viscosity was determined to be 2.80 $\pm$ 0.02mPa s. The osmolarity of this solution was determined to be 541 $\pm$ 14 mOsm/kg. The buffer capacity of the aqueous nasal spray solution was determined from linear fit of the titration curve (Fig. 6) between pH 3.5 and 5.0. The buffer capacity was determined to be 0.016 M. These results show

that the nasal spray solution is a low viscosity, somewhat hypertonic solution with adequate buffer capacity to maintain constant pH during storage.

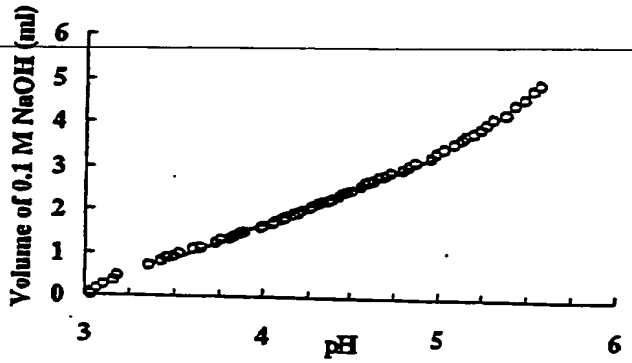


Fig. 6. Titration curve of the midazolam nasal spray. The line represents linear fit of data between pH 3.5 and 5.0.

16. That the foregoing experiments show that ring-opening occurs over time and under particular pH conditions and thus is not always present in the aqueous cyclodextrin complexation media for benzodiazepines.

17. That I consider an inherent characteristic to be one which is essential and intrinsic and thus always present and that on that basis I do not believe that ring-opening can be intrinsic in the prior art cyclodextrin complexation of benzodiazpines.

18. That I further point out that ring-opening is clearly an unrecognized and unappreciated phenomenon in the cyclodextrin complexation of benzodiazepines, so motivation to select particular conditions which produce ring-opening to enhance complexation is missing.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 23 April 2003

THORSTEINN LOFTSSON

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(016914-039)

Patent Application
Attorney's Docket No. 016914-007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Thorsteinn LOFTSSON et al	Group Art Unit: 1623
Application No.: 09/250,185	Examiner: Lawrence E. Crane
Filed: February 16, 1999	Confirmation No.: 1768
For: HIGH-ENERGY CYCLODEXTRIN COMPLEXES	

SECOND DECLARATION OF THORSTEINN LOFTSSON PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, THORSTEINN LOFTSSON, declare as follows:

1. That I am a citizen of Iceland residing at Sorlaskjol 44, IS-107 Reykjavik, Iceland.

2. That I received my M.S. Pharm. degree from the Royal Danish School of Pharmacy in Copenhagen in 1975, and my M.S. and Ph.D. degrees in 1978 and 1979, respectively, from the Department of Pharmaceutical Chemistry at the University of Kansas in Lawrence, Kansas.

3. That I have authored or co-authored over 160 papers in peer-reviewed journals, 10 book chapters, 20 patents and patent applications and about 180 abstracts.

4. That in 1998 I became a Fellow of the American Association of Pharmaceutical Sciences.

5. That I have received the following honors, awards and recognition: ~~Fulbright Fellowship (1976-1979), NATO Awards in 1978 and 1980, The Nagai~~ Foundation Tokyo, International Scholarship in 1998 and the 1992 Faculty Research Award, Faculty of Medicine, University of Iceland.

6. That I have served as a board member and a referee for numerous scientific journals and am currently a member of the editorial board of *International Journal of Pharmaceutics*, *Die Pharmazie* and *STP Pharma Sciences*.

7. That since 1979 I have been employed by the University of Iceland, Department of Pharmacy, Reykjavik, Iceland, as an Assistant Professor from 1979 to 1983, as an Associate Professor from 1983 to 1985, and as Professor of Physical Pharmacy from 1986 to the present.

8. That I have also been an Adjunct Associate Professor in the Department of Medicinal Chemistry, College of Pharmacy, University of Florida, Gainesville, Florida, from 1980 to 1987, and an Adjunct Professor, Center for Drug Discovery, College of Pharmacy, University of Florida, Gainesville, Florida, from 1987 to 1996.

9. That I am one of the inventors of the above-referenced patent application.

10. That I am familiar with the prosecution history of this application, including the prior art relied upon by the Examiner.

11. That I am making this declaration to address the Examiner's request for evidence of ring-opening as well as his assumption that ring-opening is inherent in the prior art.

12. That I have specifically considered the disclosure of the following: Loftsson and Brewster, *Pharm. Technol. Eur.*, 9(5), 26-34 (May, 1997), also referred to as Loftsson et al **AR**, and references cited therein and numbered **12, 14, 15, 16** and **18-20**, as well as Stella et al United States Patent No. 5,134,127, the teachings of which I summarize as follows:

(a) None of the prior art acknowledges the existence of the ring-opened form of benzodiazepines during complexation of benzodiazepines with any cyclodextrins or during administration of such complexes.

(b) None of the prior art mentions the contribution of the ring-opened form to the overall solubility of the benzodiazepines.

(c) No attempts were undertaken in the prior art to detect the ring-opened form of the benzodiazepines in cyclodextrin complexes.

13. That I consider an inherent characteristic to be one which is essential and intrinsic and thus always present, so that the fact that ring-opening only occurs over time and under particular circumstances means to me that it is not inherent in the prior art. It is also clearly unrecognized and unappreciated in cyclodextrin complexation by the prior art, so motivation to select particular conditions which produce ring-opening to enhance complexation is missing.

14. That to support my position, I submit the following comments and data, where all experiments referred to were conducted under my supervision and control:

Quantitative determination of benzodiazepines by high-performance liquid chromatography

Due to rapid equilibration between un-ionized and ionized species of ionizable drugs, it is not possible to separate different ion forms of a drug by reverse-phase HPLC chromatography. Even if the pH of the mobile phase is changed in such a way that the drug being analyzed is partly in its ionized forms and partly in its un-ionized form, in the mobile phase the drug will still be represented by one peak. The elution rate of ionizable drugs can be controlled by adjustment of pH of the mobile phase and this is frequently done when different drugs or drug metabolites are separated by HPLC (David G. Watson: *Pharmaceutical Analysis. A Textbook for Pharmacy Students and Pharmaceutical Chemists*, Churchill Livingstone, Edinburgh, 1999, pp. 243-246). However, different ionization forms will always be eluted as one peak from the column.

On the other hand, the conversion rate between the ring-opened and the ring-closed benzodiazepines is rather slow and the ring-opened forms are considerably more hydrophilic than the ring-closed forms. Thus, the ring-opened and the ring-closed forms are easily separated on reverse phase HPLC systems. In most cases, the ring-opened forms of benzodiazepines are eluted with the solvent fronts and, thus, are not observed in the HPLC system commonly used for quantitative determination of the benzodiazepines. Following are some examples.

Sample preparation:

Ten percent (w/v) solution of 2-hydroxypropyl-β-cyclodextrin (HPβCD) in aqueous 0.10 M HCl solution was prepared by weighing an exact amount of cyclodextrin into a volumetric flask and dissolving in aqueous 0.10 M HCl. Samples were prepared by adding an excess of the solid drug to 5 ml of each cyclodextrin-buffer solution. The samples were shaken for 4 hours on a platform shaker. The pH of this solution was between 1 and 3 (the pH was affected by the dissolved drug). After filtration through a 0.45 μm nylon filter the concentration of ring-closed benzodiazepine was measured by HPLC, immediately after a 100-fold dilution in methanol (10 μl sample in 990 μl of methanol) and again after 100-fold dilution in methanol and subsequent equilibration for 12 hours.

HPLC system: L-6200A Intelligent Pump, a L-4250 UV-VIS Detector (operated at the wavelength indicated), an AS-2000A Autosampler and a D-2500 Chromato-Integrator (all Merck-Hitachi, Germany).

Flow rate: 1.50 ml/min

Table 1. Mobile phase composition for HPLC measurements. $t_{R\ (closed)}$ is the retention time of the ring-closed form and $t_{R\ (open)}$ is the retention time of the ring-opened form of the benzodiazepine drug.

Benzodiazepine drug	Detection (nm)	Mobile phase	$t_{R\ (closed)}$ (min)	$t_{R\ (open)}$ (min)
Alprazolam	254	Acetonitrile. aq. acetic acid (2.5% v/v) (33:67)	6.92	1.34*
Bromazepam	233	Acetonitrile, aq. acetic acid (2.5% v/v) (35:65)	3.62	1.50*
Flunitrazepam	252	Acetonitrile, aq. acetic acid (2.5% v/v) (40:60)	5.20	1.40*
Midazolam	230	Acetonitrile, aq. 4 mM phosphate with 0.1% (v/v) triethylamine, pH 7.2 (55:45)	5.28	2.68
Triazolam	221	Acetonnitrile, water (40:60)	4.00	1.90*

* Approximate retention, could not be separated from the solvent front.

Results:

The results are shown in *Figures 1 to 5*. Immediately after dilution in methanol, the diluted solution contained a mixture of the ring-opened and the ring-closed form. After equilibration in methanol for 12 hours, the solution contained mainly the ring-closed form. The ring-opened forms will only be observed if the investigator is aware of their existence.

Furthermore the results show that it is possible to separate the ring-opened forms from the ring-closed forms by preparative HPLC.

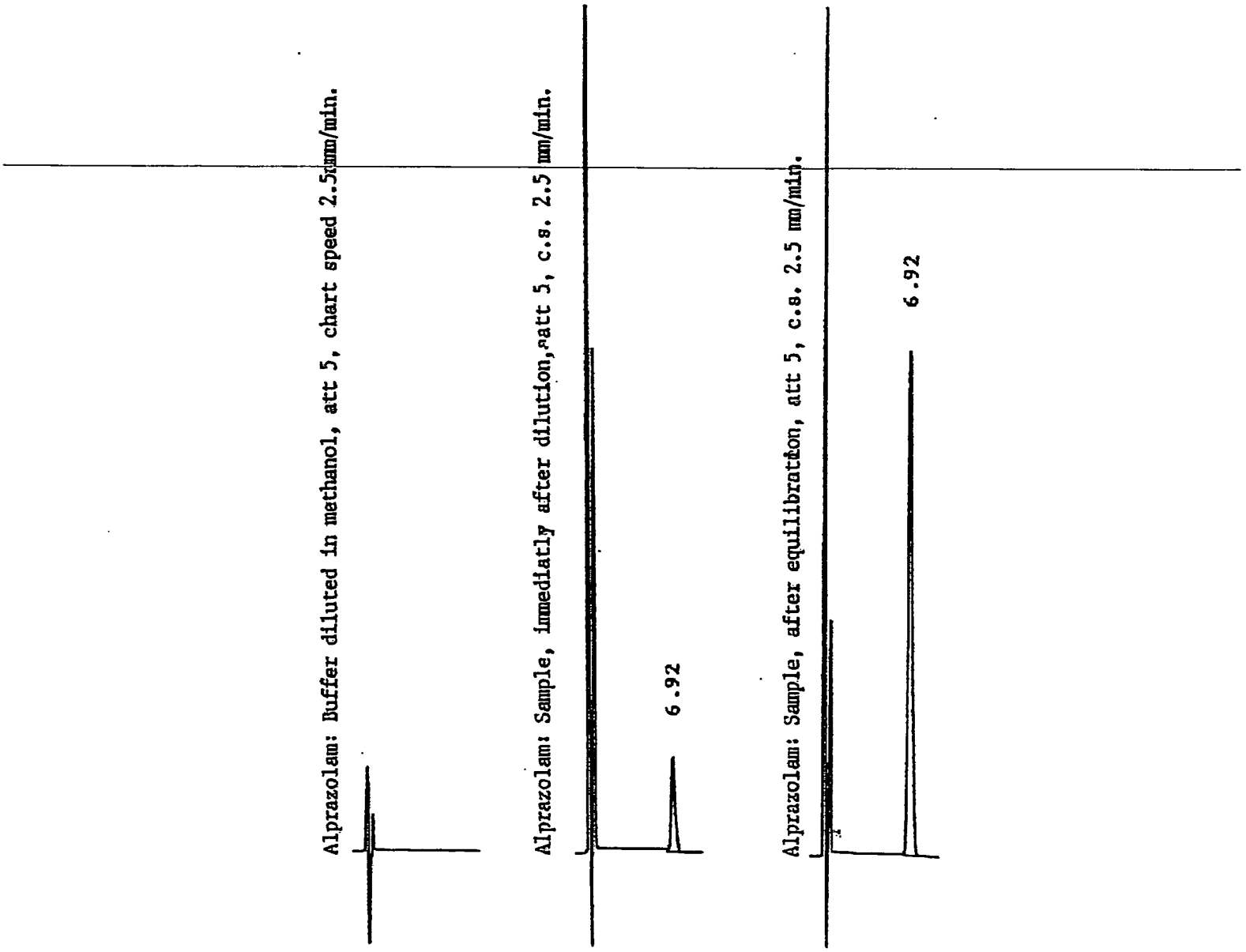

Figure 1. Alprazolam. The first chromatogram shows buffer diluted with methanol (contains no drug). The next chromatogram was obtained immediately after the sample had been diluted with methanol. The peak for the ring-opened form appears in the solvent front (at about 1.34 min) and the peak for the ring-closed form at 6.92 min. The final chromatogram was obtained after the same sample had been stored at room temperature for 12 hours. The peak of the ring-closed form has then increased and the peak of the ring-opened form (in the solvent front) has decreased. All three chromatograms were obtained under the very same HPLC conditions.

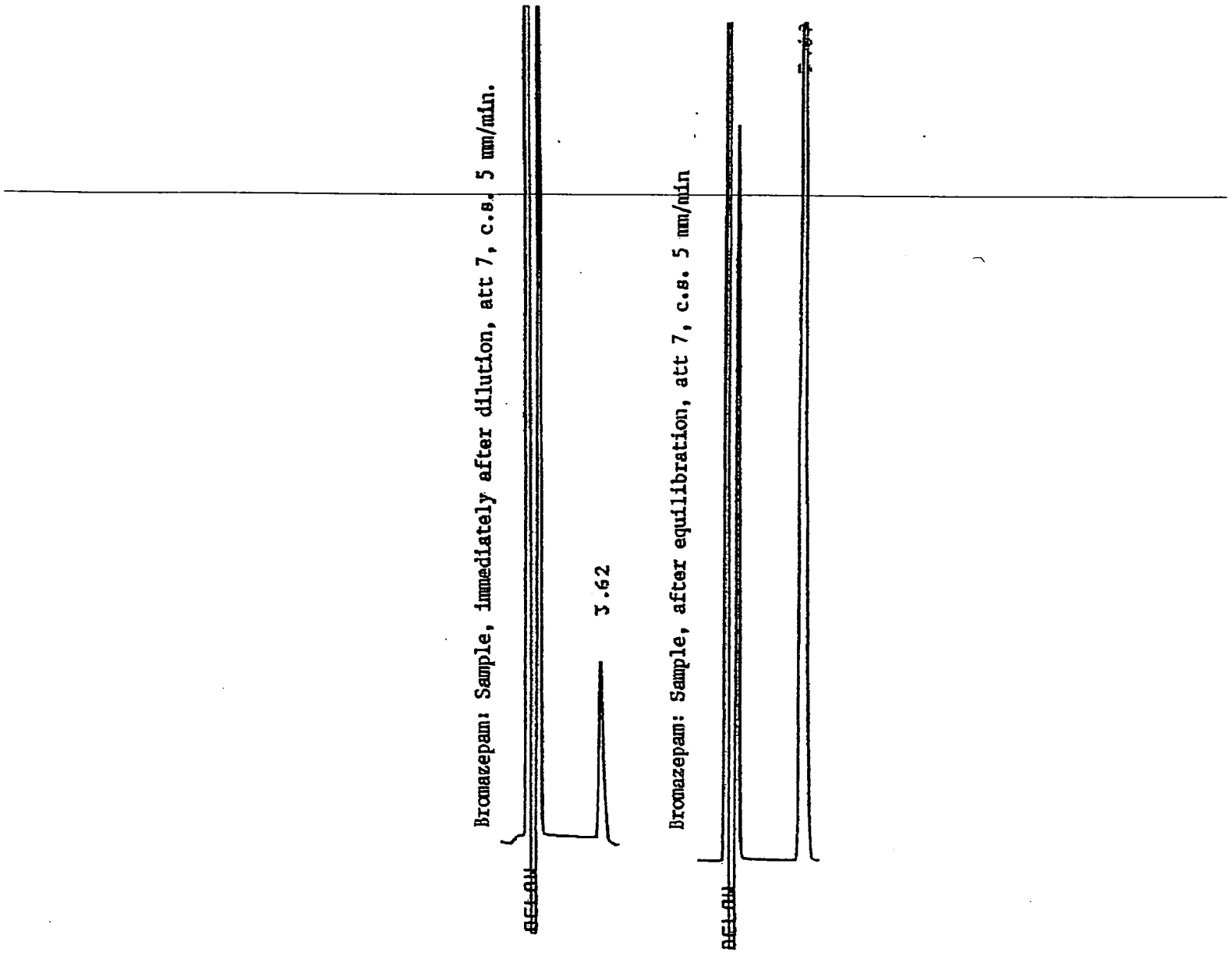

Figure 2. Bromazepam. The first chromatogram was obtained immediately after the sample had been diluted with methanol. The peak for the ring-opened form appears in the solvent front (at about 1.50 min) and the peak for the ring-closed form at 3.62 min. The second chromatogram was obtained after the same sample had been stored at room temperature for 12 hours. The peak for the ring-closed form has then increased and the peak of the ring-opened form (in the solvent front) has decreased. Both chromatograms were obtained under the very same HPLC conditions.

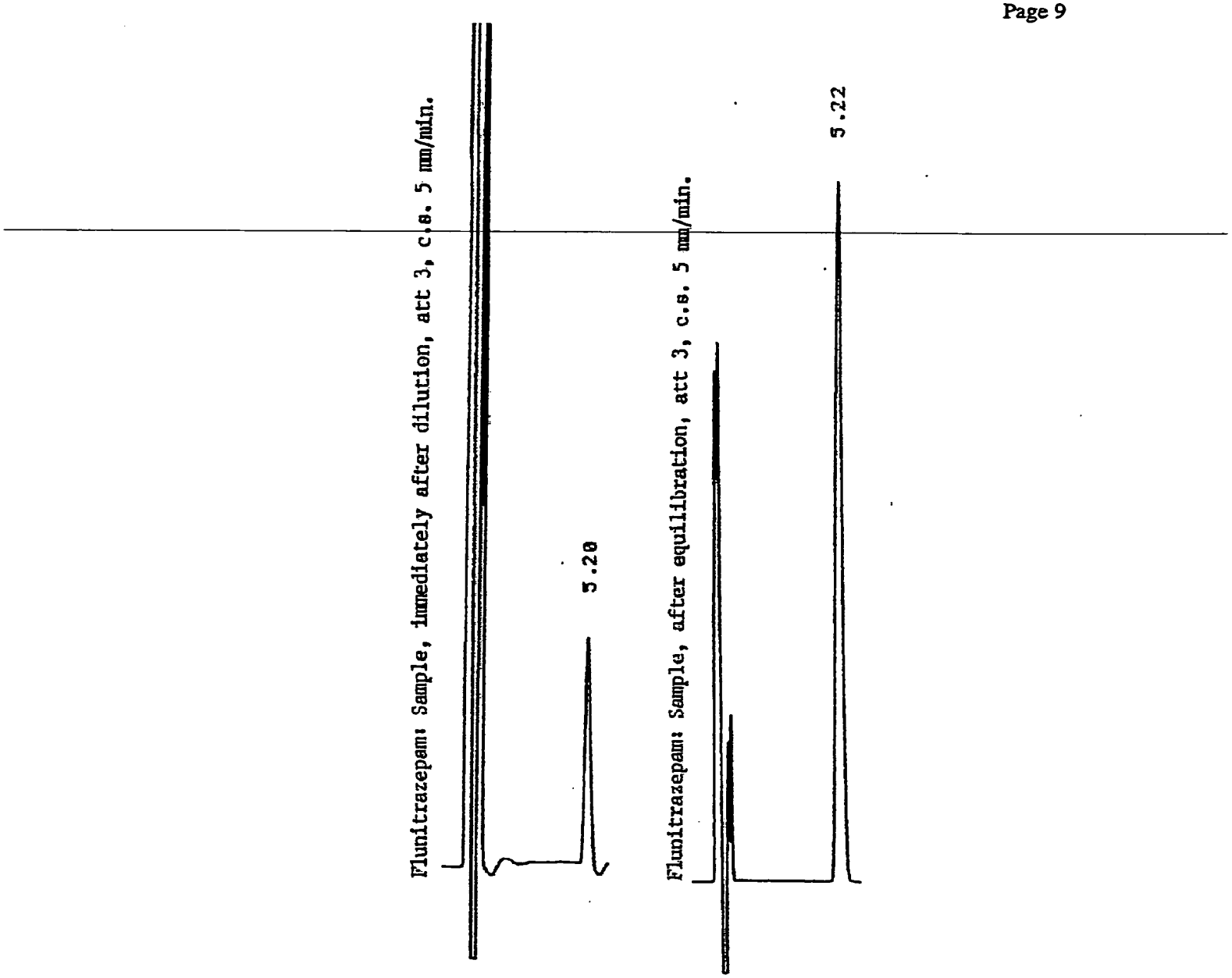

Figure 3. Flunitrazepam. The first chromatogram was obtained immediately after the sample had been diluted with methanol. The peak for the ring-opened form appears in the solvent front (at about 1.40 min) and the peak for the ring-closed form at 5.20 min. The second chromatogram was obtained after the same sample had been stored at room temperature for 12 hours. The peak for the ring-closed form has then increased and the peak of the ring-opened form (in the solvent front) has decreased. Both chromatograms were obtained under the very same HPLC conditions.

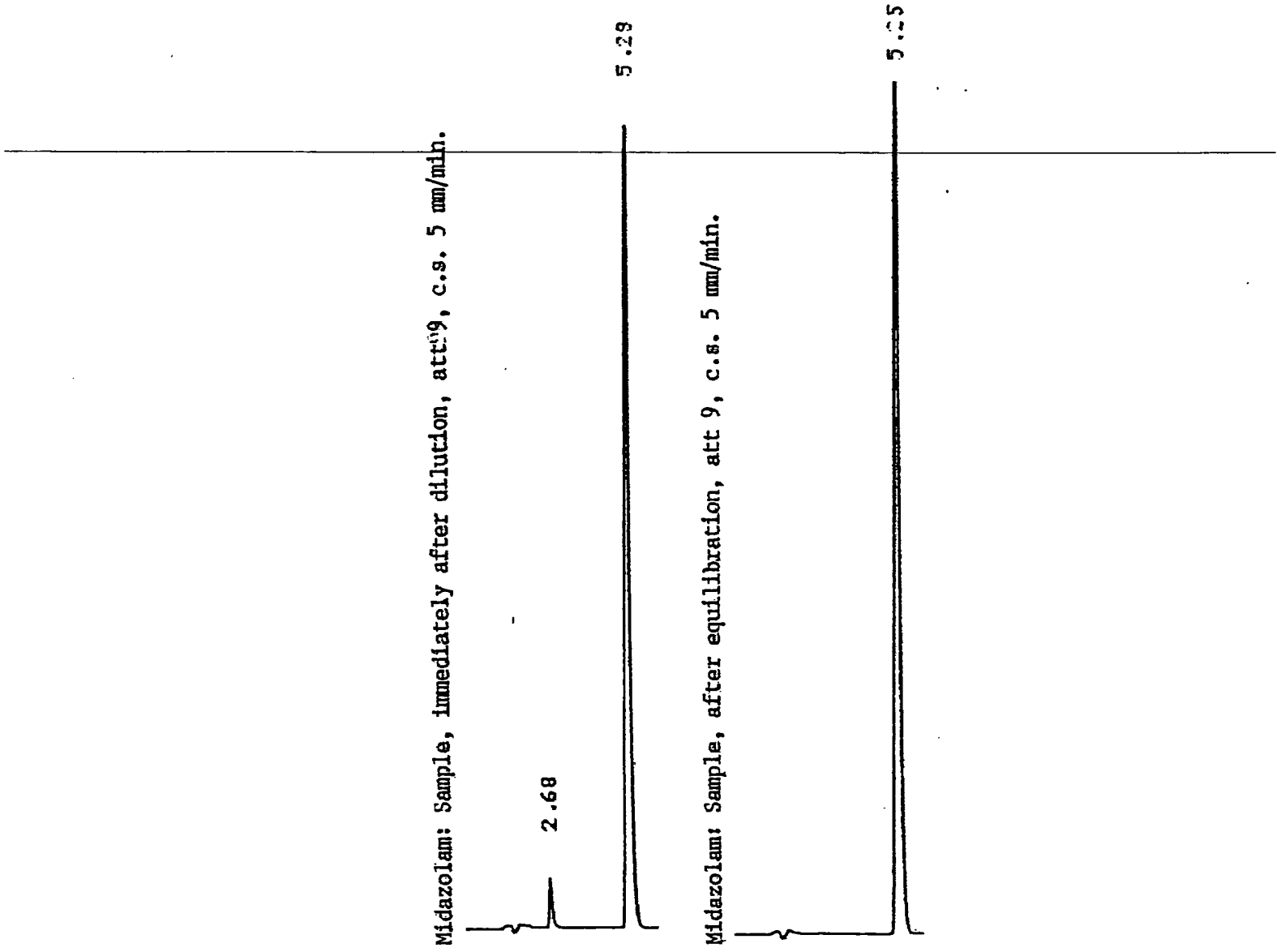

Figure 4. Midazolam. The first chromatogram was obtained immediately after the sample had been diluted with methanol. The peak for the ring-opened form appears at 2.68 min and the peak for the ring-closed form at 5.28 min. The second chromatogram was obtained after the same sample had been stored at room temperature for 12 hours. The peak for the ring-closed form has then increased and the peak of the ring-opened form has disappeared. Both chromatograms were obtained under the very same HPLC conditions.

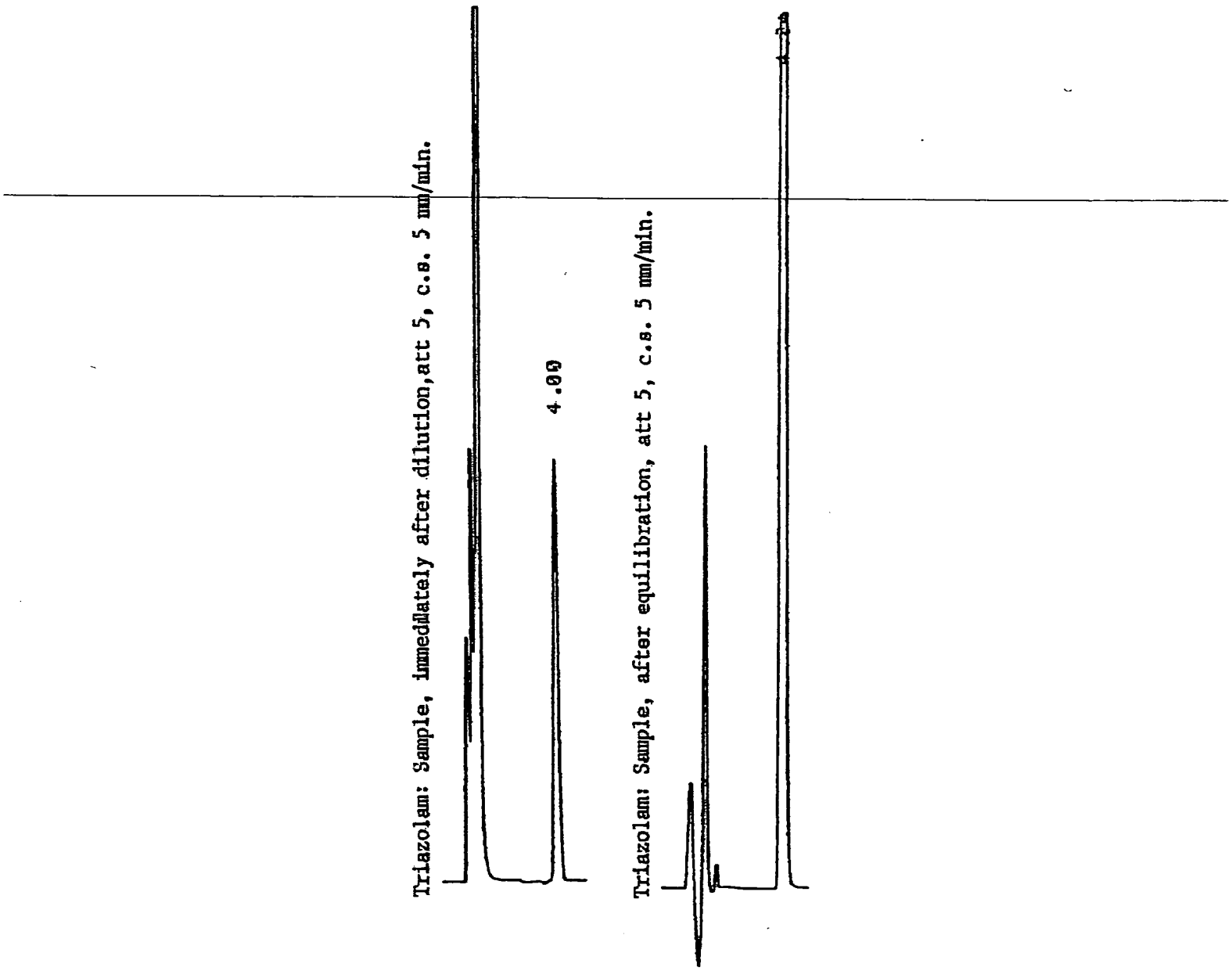

Figure 5. Triazolam. The first chromatogram was obtained immediately after the sample had been diluted with methanol. The peak for the ring-opened form appears close to the solvent front (at about 1.90 min) and the peak for the ring-closed form at 4.00 min. The second chromatogram was obtained after the same sample had been stored at room temperature for 12 hours. The peak for the ring-closed form has then increased and the peak of the ring-opened form (at the solvent front) has almost disappeared. Both chromatograms were obtained under the very same HPLC conditions.

The ring-opened form versus the total solubility

Since the conversion from the ring-opened form to the ring-closed form is rather slow, the solubility will be underestimated unless the investigator is aware of existence of the ring-opened form. Furthermore, since the ring-opened form is significantly more hydrophilic than the ring-closed form, it is usually eluted with other "impurities" at the solvent front during chromatographic analysis of benzodiazepines. Thus, the ring-opened form will be unnoticed unless the investigator makes the effort to observe it or prevent its formation. Following investigations show how important it is to be aware of both the ring-opened and ring-closed forms.

Sample preparation:

Ten percent (w/v) solutions of 2-hydroxypropyl-β-cyclodextrin (HPβCD) in different buffers were prepared by weighing an exact amount of cyclodextrin into a volumetric flask and dissolving in an aqueous buffer solution. Samples were prepared by adding an excess of the solid drug to 5 ml of each cyclodextrin-buffer solution. The samples were shaken for 4 hours on a platform shaker. After filtration through a 0.45 μm nylon filter, the concentration of ring-closed benzodiazepine was measured by HPLC, immediately after a 100-fold dilution in methanol (10 μl sample in 990 μl of methanol) and again after 100-fold dilution in methanol and subsequent equilibration for at least 12 hours. During the equilibration of the methanol-diluted sample, the ring-opened form was converted to the ring-closed form as previously shown.

Alprazolam

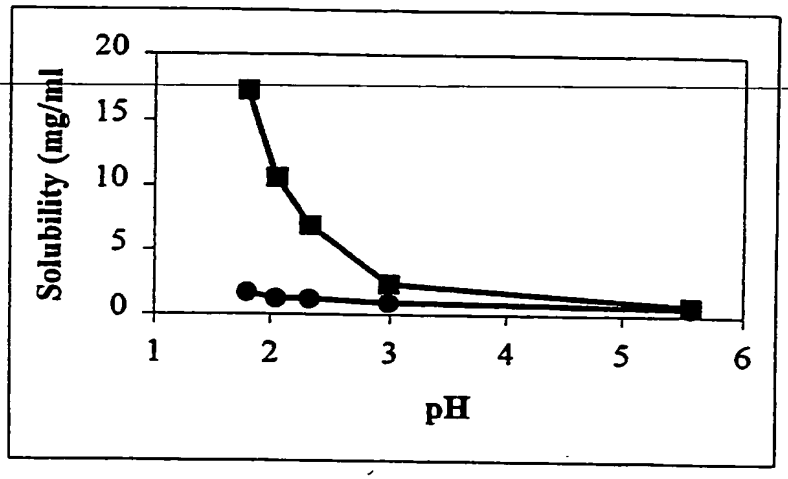

Figure 6. Solubility of alprazolam in a 10% (w/v) HPβCD solution at various pH. Ring-closed form (●); total concentration (■).

The results shown in Fig. 6 demonstrate that lowering the pH causes negligible increase in the solubility of alprazolam in aqueous 10% (w/v) HPβCD solutions if the contribution of the ring-opened form is ignored.

Bromazepam

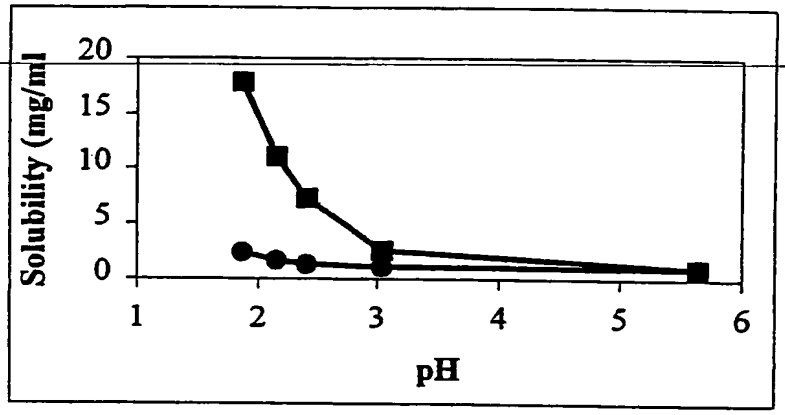

Figure 7. Solubility of bromazepam in a 10% (w/v) HPβCD solution at various pH. Ring-closed form (●); total concentration (■).

The results shown in Fig. 7 demonstrate that lowering the pH causes negligible increase in the solubility of bromazepam in aqueous 10% (w/v) HPβCD solutions if the ring-opened form is ignored. Thus, simple ionization of the ring-closed form does not result in significant enhancement in the total solubility of bromazepam.

Flunitrazepam

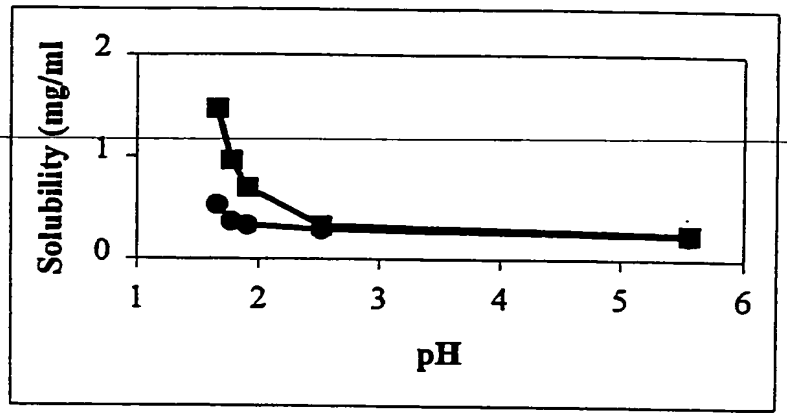

Figure 8. Solubility of flunitrazepam in a 10% (w/v) HPβCD solution at various pH. Ring-closed form (●); total concentration (■).

The results shown in Fig. 8 demonstrate that lowering the pH causes much less increase in the solubility of flunitrazepam in aqueous 10% (w/v) HPβCD solutions when only the amount of dissolved ring-closed form is determined.

Triazolam

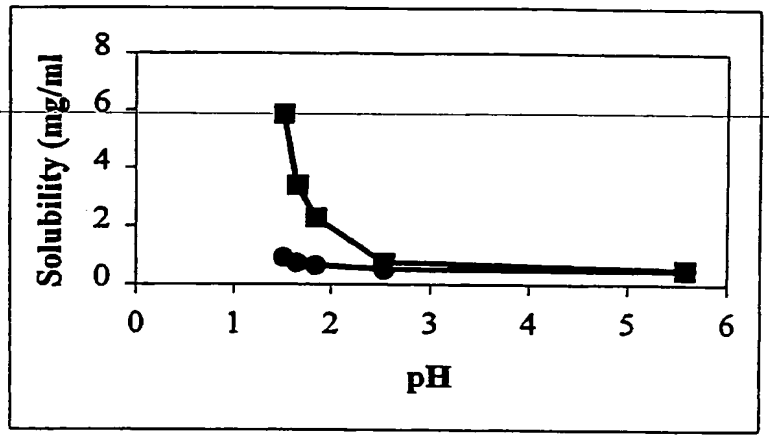

Figure 9. Solubility of triazolam in a 10% (w/v) HPβCD solution at various pH. Ring-closed form (●); total concentration (■).

The results shown in Fig. 9 demonstrate that lowering the pH causes much less increase in the solubility of triazolam in aqueous 10% (w/v) HPβCD solutions when only the amount of dissolved ring-closed form is determined.

All the data show that the contribution of the ring-opened form to the total solubility of benzodiazepines in aqueous cyclodextrin solutions will be overlooked unless the investigator is aware of the ring-opened form and makes special effort to either convert the ring-opened form to the ring-closed form before quantitative determination of the benzodiazepine or performs separate quantitative determination of the ring-opened form.

None of the cited prior art mentions the ring-opened forms of benzodiazepines in cyclodextrin complexation or even hint that there is any awareness of their existence. The ring-opened forms cannot be quantitatively determined by the analytical methods applied in the cited prior art and, thus, the ring-opened forms remained unnoticed.

15. That my conclusions from the data presented above, together with that presented in the accompanying FIRST DECLARATION OF THORSTEINN LOFTSSON PURSUANT TO 37 C.F.R. §1.132, are as follows:

(a) Ionization is an instantaneous process and thus the different ionization forms of benzodiazepines in cyclodextrin complexation media cannot be separated by analytical methods commonly applied for quantitative determination of drugs, such as reverse phase high performance chromatography (HPLC).

(b) Ring-opening (and ring-closing) of the benzodiazepine ring in cyclodextrin solution is a rather slow process. When the ring-opened form is placed under conditions that regenerates the ring-closed form (elevated pH, non-aqueous conditions etc.), it takes several hours or days for it to be fully converted to the ring-closed form.

(c) In addition to the un-ionized form, the ring-closed form of benzodiazepines can exist in at least two different ionization forms and the ring-opened form can also exist in two different ionization forms in cyclodextrin media. Since the process of ring-opening and ring-closing is a rather slow process, it is possible to observe all the 5 different forms of the benzodiazepines in cyclodextrin solution.

(d) Furthermore, the HPLC data presented shows that it is readily possible to separate the ionized ring-closed forms from the ring-opened forms by preparative HPLC.

This shows that the ring-opening is not an inherent part of the ionization of the benzodiazepine ring.

(e) As is apparent from their structures, the ring-opened forms of benzodiazepines are much more hydrophilic than the ring-closed forms and thus they are frequently eluted by HPLC within or close to the solvent front. Various degradation products, buffer salts and other media constituents are also eluted close to or within the solvent front. Thus the ring-opened forms will not be noticed unless arrangements are made to detect them.

(f) At pH below about 4 to 5, the ring-opened forms make a significant contribution to the overall solubility of benzodiazepines in cyclodextrin complexation media. Thus, the aqueous solubility of benzodiazepines will be underestimated unless specific notice is paid to the ring-opened form. At low pH, the prior art will underestimate the total solubility of benzodiazepines in aqueous cyclodextrin solutions.

(g) We have shown that the enhancement of total solubility of benzodiazepines at low pH in cyclodextrin solution can be related to formation of the diprotonized ring-opened form.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 23 April 2003

THORSTEINN LOFTSSON